CHARACTERIZATION OF AMBER MUTANTS IN THE HEXOSE PHOSPHATE UPTAKE SYSTEM OF *E. COLI*

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CHARACTERIZATION OF AMBER MUTANTS IN THE HEXOSE PHOSPHATE UPTAKE SYSTEM OF *E. COLI*

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PREFACE

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NOMENCLATURE

α-gp	sn-glycerol-3-phosphate
BSA	bovine serum albumin
CDP	cytosine diphsophate
CPM	counts per minute
cysI	gene for sulfite reductase
fructose-1-P	fructose-1-phosphate
fructose-6-P	fructose-6-phosphate
g	gram(s)
galT	gene for galactose-1-phosphate uridyl transferase
glpT	gene for glycerol phosphate transport
gltC	gene for control of glutamate transport
glucose-1-P	glucose-1-phosphate
glucose-6-P	glucose-6-phosphate
KDO	2-keto-3-deoxyoctulonate
μC	microCurie
mg	milligram
μg	microgram
ml	milliliter
mM	millimolar
μM	micromolar

NANA	N-acetylnumaminic acid
PIPES	piperazine-N-N'bis(2-ethane sulfonic acid)
SDS	sodium dodecyl sulfate
TRIS	Tris(hydroxymethyl)aminomethane
tyrT	gene for suIII suppressor (amber mutation)
uhp	gene for hexose phsophate uptake system

CHAPTER I

INTRODUCTION

The primary goal of this project was to identify and characterize mutants of Escherichia coli in the hexose phosphate uptake system (uhp) which were lacking component(s) for transport. Mutants which lacked the component(s) for transport were used in two ways. First, lesions resulting in the lack of the component(s) were mapped and the location of a structural gene for the system was established directly. Secondly, mutants lacking this transport component were used to identify the component by comparisons with the control strains.

CHAPTER II

BACKGROUND

The cell membrane is the point at which each cell regulates the metabolites which enter and leave its boundaries. As such, the membrane is the site of many regulatory processes. Active transport is one of the most important regulatory processes carried out by the membrane. Active transport requires energy to build a gradient of substances being transported.

A great deal of controversy has centered on the means by which energy is coupled to transport. One of the first theories was that ATP was the energy source. Kaback (1) suggested that electron transport powered metabolite transport using lactate dehydrogenase. Most workers now agree that some form of the chemiosmotic hypothesis of Mitchell is valid, as it is more consistent with the accumulated data for a number of systems than other suggestions. Hamilton (2) has reviewed chemiosmotic coupling to transport. In the chemiosmotic hypothesis, the protonmotive force drives transport. It is composed of components due to the pH difference across the membrane and the electrical potential due to the various ion concentration gradients across the membrane, resulting from the membrane's differential permeability to various substances. This can be represented mathmatically by $\Delta p = \Delta\Psi \frac{RT}{F} \Delta pH$.

Attempts have been make to categorize the various transport systems based on common features. Berger and Heppel (3) have used

various amino acid transport systems of E. coli to group the systems into two classes. One class has binding proteins, and is coupled to ATP, while the other has no binding proteins and is coupled to the protonmotive force.

Rottenberg (4) has discussed models of chemiosmotic transport systems in terms of the driving force at low pH (5.5). The net charge of the substance being transported must not be negative because of the electrical potential which is negative on the interior of the cell. Neutral species are transported with a proton and positive species are transported with their native charge, thereby making transport of these species electrogenic (with net charge transfer across the membrane) and allowing for coupling to the membrane potential. Negatively charged species must be neutralized to be transported at all. This will result in electroneutral transport (with no net charge transfer) which cannot be coupled to the electrical potential, but is coupled to the pH gradient. In work which substantiates these models, Ramos and Kaback (5-8) have also found that those systems coupled to the pH gradient vary the ratio of substrate:protons in response to the external pH. All systems will be driven by the electrical potential at pH's above neutral.

Other Systems

The Phosphotransferase System

One of the early systems studied was the phosphotransferase (Roseman) system (reviewed {9}). The system catalyzes phosphoenolpyruvate-dependent phosphorylation and transport of several hexoses (10). The

general mechanism follows.

$$\text{PEP} + \text{HPr} \longrightarrow \text{pyruvate} + \text{P-HPr}$$

$$\text{P-HPr} + \text{glucose} \longrightarrow \text{HPr} + \text{glucose-6-P}$$

$$\text{PEP} + \text{glucose} \longrightarrow \text{pyruvate} + \text{glucose-6-P}$$

Soluble components are Enzyme I (EI) and HPr. The membrane bound complex is called Enzyme II (EII) and is made of IIA which has a specific component for each of several hexoses carried by the system, and IIB. A and B are both required for phosphorylation. IIB has been isolated and has a molecular weight of 36,000 and is a major constituent of the membrane.

Periplasmic Transport Components

Many proteins are located between the inner and outer membranes of gram negative bacteria and are released on osmotic shock from this space (reviewed {11}). Some of these proteins have enzymatic activities, and others are components of transport systems. Many of these transport components have the ability to bind their substrates. Several of the best studied binding proteins are galactose, sulfate, leucine and histidine. In each case mutants have been found which have an altered binding protein and a defect in transport. Revertants from these strains having transport abilities, also have regained functionality in the periplasmic binding proteins. Osmotic shock also leads to loss of transport ability which can be restored by addition of the binding protein in some cases.

A periplasmic protein involved in the transport of α-gp has been identified (12). This protein is required for transport but is dif-

ferent in that it does not bind α-gp. Strains normally show α-gp transport in membrane vesicles, even though the periplasmic protein (the glpT gene product) is not present in the vesicles in any of the strains. The proposed role of the glpT gene product is to help overcome the diffusion barrier set up by the outer membrane. The action in vesicles is not easily explained, unless the gene glpT is responsible for several gene products, or the periplasmic protein is located in both the inner membrane and the periplasmic space.

The histidine J protein, a binding protein of S. typhimurium, interacts with histidine and a membrane protein (the P protein) to accomplish transport. The P protein interacts with several binding protein-substrate complexes and thus is common to several transport systems. It was found in some mutants that the J protein shows a higher molecular weight than the functional protein does (13). Peptide mapping showed the added weight was in the interior of the protein sequence. Post-translational modification was postulated as the mechanism for the molecular weight modification.

The Lactose System

The most thoroughly studied transport system is the lactose system in E. coli which transports lactose by means of a proton symport (14). The M protein is the recognition site for this system, and is located in the membrane (15). It is a hydrophobic protein with a molecular weight of 30,000 as shown by SDS acrylamide gel electrophoresis. The method of identification of the M protein was novel, and is simplified here and only the basis for identification is discussed. An essential sulfhydryl on the M protein can be protected from reaction with N-

ethylmaleimide, a non-transportable substrate analog. All non-protected sulfhydryls can be blocked with unlabeled N-ethylmaleimide. Removal of the non-transportable substrate analog and reaction of the protected sulfhydryl with radioactive N-ethylmaleimide results in labeling only the protein involved in transport. This procedure provided a means of detection of the protein during purification procedures. This is one approach to the problem of a lack of an assay in purification procedures. This is one approach which yields an inactive protein which has been covalently labeled in the active site region, and has not been pursued in most systems.

Kolber and Stein (16) used column chromatographic separations to detect components of the lactose system by comparisons of induced, uninduced, and constitutive strains. In a variant of this approach using SDS acrylamide gel electrophoresis, the M protein has been found in the inner and outer membranes (17). This study also demonstrated that 10% of the β-galactosidase is located with the inner membrane.

The mechanism of lactose transport in normal, energized cells, involves binding of proton(s) and substrate to the carrier, on the exterior of the cell. Translocation occurs in response to the proton-motive force, and then the proton(s) and lactose are released into the cell. Transport in this system is an electrogenic process which acts to dissipate the electrical potential and pH difference across the membrane. Koch (18) found that the carrier actually has a reduction in affinity for substrates on the internal face of the membrane compared to that on the external face of the membrane. An explanation for this in light of the chemiosmotic hypothesis, is that a lowered internal pH results in a reduction in the transport capacity. In starved cells

which have been depleted of energy, facilitated diffusion (equilibration of the concentration gradient across the membrane) of lactose is only possible in the presence of an exogenously added energy source such as glucose or succinate, or an uncoupler (19). In these cells, translocation cannot occur because the extrusion of proton(s) is impossible. In starved cells, the introduction of a membrane-permeant ion like thiocyanate, or valinomycin (allowing the membrane to become permeable to K+) to compensate for the charge translocation across the membrane or addition of an uncoupler to allow proton movement, allows equilibration of substrate across the membrane to occur (14). Thus, facillitated diffusion requires compensation for the charge movement which occurs during transport.

Kaback's group has used dansylated galactoside derivatives to show that there is a change in fluorescence intensity and fluorescence polarization upon substrate binding to the carrier in membrane vesicles (20,21). In the same type of study, photoactivated lactose analogs were used to inactivate and label the system (22-23). Increased binding occurs if membranes are energized with D-lactate. This is in membranes prepared according to Kaback's EDTA-lysozyme method, which have the same orientation as intact cells. Energization probably affects the relative proportion of a high-affinity form of the carrier present on the outside of the cell to the amount of carrier present on the interior of the cell in a low affinity form (21). Kennedy (24) has used reversed oriented vesicles and has shown that direct binding can be measured with no energization, although it is reduced. Based on these observations if true "cycling" of the carrier was present, it would seem that there would be a mixture of states ("in" and "out") in vesi-

cles of both orientations. The isolation procedures may act to reorient some of the carriers, or the carrier may face one direction in a "resting" state. The amounts of sugar bound per mg of membrane protein are 0.11 nmoles with the Kennedy procedure, and 1.14 nmoles with the Kaback procedure. This observation is consistent with energization providing an increase in amount of substrate bound.

Reconstitution

The newest, most innovative and generally applicable line of research to be used in the study of transport systems is reconstitution. The membrane enzyme lactate dehydrogenase has been studied a great deal in vesicles. Interest naturally centered on this enzyme because of Kaback's initial suggestion that it played a central role in energy coupling (as described elesewhere) (1). (This is not currently widely accepted as an explanation.) The enzyme has been isolated from membrane vesicles prepared by the lysozyme-EDTA method (25-27). This has shown that membrane vesicles can be used as a partial purification method for membrane proteins (i.e. separation from cellular contents and outer membrane components). Addition of purified lactate dehydrogenase to vesicles (in a type of reconstitution) lacking the enzymatic activity and unable to carry out certain transport functions which are apparently linked to dehydrogenase activity, restored enzyme activity, and transport function (28,29). Several transport systems have been examined in a reconstituted system, including those for proline (30) and lactose (31). Reconstitution involved isolation of the transport proteins using aprotic solvents (without loss of activity) and addition of these protein(s) to purified phospholipids or membrane vesicles from strains

lacking that activity. In this way the sidedness present in whole cells may be reformed in a more pure system.

The Hexose Phosphate Uptake System

The hexose phsophate uptake system (uhp) was first reported by Frankel in 1964 (32). Many hexose phosphates including fructose-1-P, fructose-6-P, glucose-1-P, glucose-6-P, mannose-6-P, and 2-deoxyglucose-6-P are substrates for the system. Sorbitol-6-phosphate (33), glucose-6-sulfate, and fructose-diphosphate (35) are not transported by the system. The system has several features which make it particularly interesting to study. These include external induction, functionality in vesicles, sensitivity to osmotic shock, an undisclosed mechanims, and unidentified conponent(s). The uhp system has been reviewed by Dietz (35).

The specificity of the system was determined using competition between unlabeled glucose-6-P or fructose-6-P and labeled glucose-6-P in a ratio of 50:1 (unlabeled:labeled) to demonstrate that a specific carrier was responsible for transport of these hexose phosphates, and that competition was possible (36). The system was shown to be saturable by measuring a range of concentrations of labeled substrate. Counterflow of substrates was also demonstrated after uptake of labeled substrate by addition of an excess of unlabeled substrate and monitoring the egress of labeled substrate.

External Induction

Winkler (37) reported that substrates of the system could also induce it. However, it was later found that only external glucose-6-P or

2-deoxyglucose-6-phosphate actually induce the system (38-40). It was found that fructose-6-P could induce the system only because phosphoglucosisomerase activity was present (33,42).

In cells with a normal pool of intracellular glucose-6-P, induction does not occur (39,41,42). In normal cells after induction, accumulation occurs until a 20-fold excess exists internally when compared to the external concentration (39,41,42). Cells which are exposed to glucose, but are blocked in glucose-6-P utilization can build up to a 120-fold excess internally but the uhp system is not induced (39). These cells, if induced by glucose-6-P added to the external medium will lose glucose-6-P until only a 20-fold excess exists internally (39). There are two possible explanations for this external induction according to Winkler (42). Glucose-6-P as it crosses the membrane could be the true inducer. Because glucose-6-P seems to be unaltered in crossing the membrane, a difference in the glucose-6-P coming from the outside of the cell and that originating inside would be necessary, which seems unlikely because of the complexity involved. The other alternative would be a membrane-associated induction-repression system which is in contact with a DNA complex that induces the system. A specific note should be made that a membrane-associated component must recognize glucose-6-P even in the uninduced state according to this model.

Osmotic Shock

The uhp transport system is one of many transport systems sensitive to osmotic shock (11). Many systems release periplasmic binding proteins upon this shock (reviewed above). No such uhp component has

been found by looking for binding activity (Essenberg, unpublished, 35). This lack of a protein, coupled with other work to be described, implies that the transport component(s) must be in the membrane.

Mutants

The uhp system is shown in Figure 1. The complete E. coli K12 linkage map and symbols are shown in Bachman, Low and Taylor (43). Negative and constitutive mutants have been made by several different methods. Kornberg and Smith (44) made mutants in phosphoenolpyruvate carboxylase activity. This enzyme is required for maintenance of C4 acids in the tricarboxylic acid cycle. The mutants grew on acetate, using the glyoxylate cycle, but not on other carbohydrates. Isocitrate lyase is the key enzyme in the glyoxylate cycle and is inhibited by C3 acids. By growing cells on hexose phosphates and acetate, (with the hexose phosphates contributing to the C3 acid pool) uhp- mutants may be selected. Constitutive mutants have been selected on the basis of growth on fructose-1-P (45). Fructose-1-P cannot induce the system, therefore, cells growing on it must have the system constitutively present. Kadner and Winkler (46) isolated uhp- mutants by ethyl methane sulfonate mutagenesis followed by penicillin selection. Other mutants were selected using resistance to fosfomycin in a direct selection. (Fosfomycin is an inhibitor of N-acetyl glucosamine conversion to N-acetyl muramic acid so proper cell envelope formation is not possible.) Fosfomycin enters the cell via either the glycerol phosphate uptake system or the uhp system, so mutants may be isolated by growing on an appropriate carbon source and fosfomycin. Kadner (47) selected constitutive mutants by growth on fructose-6-P in the same

Figure 1. The uhp Region

manner as described above for fructose-1-P. Ethyl methane sulfonate or nitrous acid mutagenesis followed by penicillin selection was used by Essenberg and Kornberg (48) to isolate negative and constitutive mutants.

Kornberg and Smith (44) and Ferenci et al. (45) mapped their negative and constitutive mutants and found them to be near minute 81. The mutants were found to be 50% cotransducible with pyrE. Transductions between constitutive and negative mutants showed them to be inseparable by the crosses. Kadner and Winkler (46) divided negative mutants into two groups, one in a regulatory gene and the other in a structural gene, with the structural gene lying nearer pyrE. The differentiation between the two genes was based on the phenotypes of the revertants of the mutants. Revertants of the structural gene were expected to give only wild type inducibility while some of the revertants of the regulatory gene could exhibit altered regulation. In this case the regulatory mutants showed temperature sensitive growth due to temperature sensitive synthesis or constitutive uptake of glucose-6-P. Essenberg and Kornberg (48) examined several mutants and determined the cotransduction frequency of uhp and gltC to be 50-90%. They mapped their mutants in relation to other markers (giving the map shown in Figure 1) and in relation to each other. They found that a constitutive mutant lies on the opposite side of the negative uhp lesions from pyrE. This agrees with Kadner's (47) work.

There is only a tentative location of the structural gene region, based on negative evidence. The structural gene region should be located by direct methods. The best way to locate the structural region unequivocally and directly would be to isolate mutants which can be

shown to be uhp- due to a mutation which eliminates protein production (e.g. nonsense mutants). Even with this evidence a control element could be located this way if there were a protein produced (i.e. an activator, a repressor, or a recognition site at the cell surface, not active in the transport system but required for induction).

Energy Coupling

Pogell et al. (33) first noted in induced cells which had been stored at 4^{o}C. for 2 days that an energy source was required to allow them to take up labeled 2-deoxyglucose-6-phosphate. Winkler (36) found the uhp system to be inhibited by uncouplers and inhibitors of oxidative phosphorylation. Uncoupled transport of glucose-6-P could be inhibited by fructose-6-P. Energy coupling reduced the affinity of the carrier (36). Based on this evidence, Winkler suggested that the affinity of influx is increased by energy coupling. This is in contrast to the β-galactoside system in which the affinity of efflux is decreased with energy coupling. In experiments measuring labeled glucose-6-P uptake and changes in external pH of a concentrated cell suspension, Essenberg and Kornberg (49) reported the system was a proton symport (cotransport of glucose-6-P and proton(s)). Using inhibitors they (49) showed that energy could come from either ATP or electron transport.

Dietz (50) first demonstrated that the uhp system functioned in vesicles. Using membrane vesicles and measuring the differences in pH and electrical potential across the membrane by using compounds which distribute themselves across the membrane in response to the potential, Ramos and Kaback (5-8) have demonstrated that the uhp system is coupled

only to the difference in pH across the membrane at pH 5.5, but is coupled to the electrical potential at pH 8.0. Related findings (6-8) showed that the relative stoichiometry between hexose phosphate and proton(s) entering via the symport mechanism is altered depending upon the external pH.

Results with membrane vesicles have proven that the uhp system functions in a physiological manner in response to inhibitors, and the Km and v_{max} are similar to those for the system in whole cells. This provides the best evidence that the components necessary for physiological function of the uhp system are present in membrane vesicles, and therefore are present in the inner membrane.

Possible Approaches and the Current Study

Types of Mutants

The major goal of this research was to isolate and characterize mutants in the uhp system which were lacking a required component for transport.

Several types of mutations will lead to the desired lack of a protein product and would be appropriate for this study. Deletion mutants for a particular gene give no expression of that gene because they lack that genetic information. There is no guarantee that a deletion will be in a structural gene. These mutants are not leaky or able to revert. There is a possibility of having genes missing other than the gene in question, depending upon the size of the deletion. Because reversion is impossible, no method of reversion or suppression of the mutation is available in these mutants.

Mu phage (51) may insert into any (structural or control) gene. Again, there is no reason to believe that these insertions will be in a structural gene. Mu insertions also do not revert and thus have the same assets and liabilities as do deletion mutants. The possibility of multiple insertions in the same vicinity should be very remote.

Another possible type of mutation is a nonsense mutant. These mutants have a base change in the sequence of a protein-producing gene causing one of the three possible termination codons used in protein synthesis to be encountered before the normal end of the protein. Fragments may result from partial synthesis, but probably will be degraded faster than normal proteins. (For a review, see Goldberg and Dice {52}). Nonsense mutants are suppressed on introduction of a suppressor- either an intragenic mutation which "corrects" the lesion or a nonsense suppressor at a second site. (The possibility of an intragenic mutation makes it necessary to confirm that suppression of the mutation after the introduction of suppressors is due to the suppressor, and not an intragenic mutation. A suppressor is an altered t-RNA which recognizes a termination codon because of a base change in the anticodon, and inserts an amino acid in place of the termination codon.) In this way, controls (strains which have a positive phenotype in the uhp system) exist both before and after the introduction of the mutation in the form of the parent and the suppressed amber mutant strains. This is advantageous because of the need to prove that the observed mutation(s) are due to a single amber mutation. The parent contains the activity and the suppressed strain has regained at least partial activity through the suppressor's recognition of the termination codon corresponding to an amino acid. Multiple amber mutations in one region are

much less likely than deletions of multiple genes in the same region. Any of the mutants discussed could result in a control gene mutation which might cause the lack of structural protein(s). This type of mutant could successfully be used to look for components for the system, but would result in the mapping of a control gene, not a structural gene. Any of these mutations may be polar (result in less synthesis of some proteins because of premature release of the protein synthesis apparatus and lack of good ribosome binding sites after the mutation) if the mutation is early in a given region under the same genetic control. If polar mutants were obtained, proteins produced later in the sequence of DNA under the same genetic control would show reduced quantities. These proteins could be identified in this case.

Considering the above discussion and the avilability of appropriate strains, nonsense mutants were chosen for this study. Several suppressors were available for the study. The SuIII recognizes only amber mutations and has a higher recognition rate than some of the other possible suppressors, and so was chosen. Only one suppressor was used in this study. When the suppressor is introduced by mating it appears to be more stable than when it is intruduced by transduction.

Examination of Differences in Mutants

After complete mapping of the lesions in the amber mutant strains, mutants in the affected gene(s) can be compared to the controls to identify the protein product of the affected gene(s). Because a transport protein is being sought, the location of the protein can be assumed to be at or near the exterior surface of the cell. As mentioned previously, search of the periplasmic space has not yielded a hexose phos-

phate binding activity which correlates with the uhp system (Essenberg, R. C., unpublished results, 35). This is not conclusive proof for the lack of a periplasmic component(s). A report of location of a component for the glycerol phosphate transport system using various types of mutants including amber mutants, has shown that the component has no binding activity (12). The inner and outer membranes remain as possible locations for the component(s) of the uhp system. As was first noted by Dietz (50), physiological uptake is observed in vesicles (that contain only inner membranes in the strains which were used in the initial study). Thus, it is probable that the carrier for uhp is located in the inner membrane.

There is some evidence that outer membrane channels may be involved in bringing some substances into the periplasmic space as a first step in transport (53,54). Also, in the case of the maltose system, there are inner and outer membrane components involved in transport. If amber mutants lacking uhp activity were lacking a protein which acted to form or acted on an outer membrane channel, the protein would be located in the outer membrane. Mutants of this sort would be expected to have normal uptake in vesicles stripped of the outer membrane. An outer membrane mutant would also be expected to have altered properties in other systems as several systems may use the same channel.

To eliminate many cellular proteins from those to be examined in looking for differences in the mutants and the control strains, the membranes were isolated. Membrane isolation involves breaking the cells open (osmotically, with a French press, or sonically), removal of the whole cells, and precipitation of the membranes. Fractionation of the membranes into inner and outer membranes allowed more definative loca-

tion of the differences between the strains. There are many methods for accomplishing fractionation. The method of Osborn et al. (55) is based on the destabilization of the outer membrane with EDTA by the removal of divalent cations. Following this, penetration of the outer membrane by lysozyme and cleavage of the peptidoglycan between the inner and outer membranes facilitates loosening of the membranes from one another. After lysis and isolation of the crude envelopes, inner and outer membranes are separated by centrifugation through a sucrose gradient.

Schnaitman's (56) procedure involves disruption of the cell in the presence of Mg++, to stabilize the outer membrane. Isolation of crude envelopes (inner and outer membranes together) is the next step. Separation of the inner and outer membranes is based on the solubility of the inner membrane in Triton X-100. (The outer membrane is soluble in Triton X-100 only when the associated Mg++ is removed.)

The method of Joseleau and Kepes (57) is based on isolation of crude envelopes following the disruption of the cell and stabilization of the envelopes with Mg++. The inner and outer membranes are separated based on their electrophoretic mobility in a stabilizing sucrose gradient.

For this work, Schnaitman's (56) procedure was used because of its relative ease and rapid isolation possible. It is generally accepted that membrane proteins are stabilized by the Triton X-100 and this was considered an asset. For later work, (SDS gel electrophoresis) it was found that the Triton X-100 had to be removed. The association of Triton X-100 with these hydrophobic proteins is very close, and with the exception of the procedure outlined by Holloway (58) using hydro-

phobic polystyrene beads for the removal of Triton X-100, most methods resort to harsh prcedures to separate the proteins from the Triton X-100. The only problem with the method is that the beads used could have an affinity for the protein which is being sought. The presence of the Triton X-100 was both an asset and a liability.

After a method of isolation of the membranes was chosen, the membrane proteins in the fractions obtained had to be separated sufficiently to allow for comparisons. The method of separation must be capable of separation of hydrophobic proteins; it must be sufficiently sensitive to measure differences in minor components of the membrane fractions; and it must be capable of running multiple samples (i.e. from different strains) under identical condtions. Many procedures are available, as most standard protein separation procedures may be modified to use membrane proteins in the form of solubilized proteins in Triton X-100. SDS gel electrophoresis was chosen because of its ease and general acceptance as a high resolution technique. Slab gels gave the capability of multiple samples run under identical conditions at the same time.

Assay of transport systems using their physiological activity is nearly impossible without proper membrane structure (either in whole cells or vesicles) to distinguish "inside" from "outside". A means assay capable of being used in mixtures of solubilized proteins would be very powerful in following the activity in purification. If binding of hexose phsophates to its carrier could be measured, it could be used as an assay for that portion of the transport system.

Equilibrium dialysis is the basic method used to measure substrate binding to macromolecules. The amount of a labeled substrate bound to

protein contained in dialysis membrane is measured and compared to the amount of the substrate that is free on the outside of the tubing. A newer version of this method is flow dialysis (59). The method uses a small incubation chamber above a dialysis membrane which forms the bottom of the chamber. Buffer is pumped at a constant rate under the membrane, with a minimum volume present under the membrane. Proteins and labeled substrate are added to the upper chamber. The rate of appearance of labeled substrate in the effluent is proportional to the free concentration in the upper chamber. The results are much more rapidly obtained than in equilibrium dialysis, but the method requires high levels of radioactive substrate in the upper chamber.

Future Implications

This study's major goal was not physical characterization of the transport system, the information gained about the probable component(s) will provide the basis to begin isolation. Many studies may be accomplished on a purified system. Reconstitution in a purified state would be a powerful tool to study the actual mechanism of transport. Physical characterization of the purified components might provide insight into their integration into the membrane, which might help determine if there is a conformation change in the process of transport. Identification of component(s) of the system may indicate the mechanism of transport. Examples of possible mechaisms are channels through which substrates pass, or a binding protein, which requires interaction with another component responsible for translocation, external induction, or energy coupling.

CHAPTER III

MATERIALS AND METHODS

Materials

Ammonium persulfate was obtained from Bio Rad. ICN supplied uniformly labeled {^{14}C}-glucose-6-P of specific activity 150-300 mC/mmole, and {^{14}C}-sn-glycerol-3-phosphate at 80 mC/mmole. Dialysis membrane was obtained from Union Carbide. 0.45 um pore size nitrocellulose filters were obtained from Gelman Instrument Co. Amberlite supplied their XAD-2 beads. Bis-o-(methylstyryl)-benzene, 2,5-diphenyloxazole, Triton X-100, sodium dodceyl sulfate, acrylamide, Folin reagent, glucose-6-P, 2-deoxyglucose-6-phosphate, fructose-6-P, fructose-1-P, Coomassie Blue R, N,N'methylbisacrylamide, Tris(hydroxymethyl)aminomethane, piperazine-N-N'bis(2-ethane sulfonic acid), α-methyl glutamate, and proteins used as molecular weight standards in gel electrophoresis were obtained from Sigma. Other chemicals used were obtained in reagent grade.

Phage and Strains of Interest

Table I lists the phage and strains of E. coli used in this research. Bachman, Low and Taylor (43) reference the various gene abbreviations and show their location on the E. coli genome.

TABLE I

STRAINS OF INTEREST

Strain	Markers (References)
H12r8a	Hfr(PO2A), phoA5 (amber), rel-1, tonA22, $T2^R$, tyrT34(SuIII), pho35, (CGSC #2597) (60)
HfrH	Hfr(PO1), relA1, λ-, thi-1, spoT1, supQ80, (CGSC #259) (61)
AT2243	Hfr(PO2A), metB, pyrE, (from A. L. Taylor)
RE21	Hfr(PO2A), metB, pyrE, uhp-40, tna, (from AT2243) (48)
RE37	Hfr(PO2A), metB, pyrE, uhp-2, (from AT2243) (48)
RE74	Hfr(PO2A), metB, pyrE, uhp-40, $gltC^c$, tna, (from RE21 x ϕCS7, $gltC^c$ selection)
RE76	Hfr(PO2A), metB, pyrE, uhp-2, $gltC^c$, (from RE37 x ϕCS7, $gltC^c$ selection)
JM463	F-, proA or B, metB, $cysI^{am}$, his, fpk, glpT, uhp^c, $galT^{am}$, str, (from M. C. Jones Mortimer)
RE89	F-, proA or B, metB, $cysI^{am}$, his, fpk, glpT, uhp^c, $galT^{am}$, str, $gltC^c$, (from JM463 x ϕCS7, $gltC^c$, selection)
7	Hfr(PO2A) (from Barry Rosen)
P1 cml-clr 100	temperature sensitive lysogeny, specialized transduction for the cml region (from J. L. Rosner) (62)
T4 am N82	T4 phage which causes lysis of cells carrying an amber suppressor

Media and Conditions of Growth

Minimal salts medium 56 (63) was used at half strength with lug/ml of thiamine·HCl. Carbon sources were used at 10 mM except glycerol which was used at 20 mM and glucose-6-P which was used at 5 mM. Amino acids, purines and pyrimidines were added to a final concentration of 100ug/ml. When plates were used 2% (w/v) agar was used. LB (64) was used as a complete medium. Aeration was provided by a New Brunswick reciprocating shaker. Growth was at 37°C. except in growth of phage P1 cml clr lysogens which were grown at 30°C.

Method of Selection of Mutants

Strain RE89 was plated on glycerol minimal salts media with 1 mM fructose-1-P. Colonies appearing were tested for growth on glucose and glucose-6-P plates. Such colonies were then streaked for single colonies. A sample was then mated in liquid media with an Hfr, H12r8a, which transfers tryT, the gene for an altered t-RNA_{tyr}, that recognizes UAG (the amber termination codon). Recombinants were selected for the suppression of two known amber markers (cysI and galT) using streptomycin as a counterselection. Recombinants were then checked for suppression of the uhp lesion, and two strains whose uhp lesions were suppressed were retained and were designated RE98 and RE99. The suppressed versions of RE98 and RE99 were designated RE101 and RE102, respectively. Strains RE101 and RE102 were checked for the presence of the suppressor by crossstreaking with T4 am N82 phage which only causes lysis of cells carrying amber suppressors. Strains RE104 and RE105 were made from RE98 and RE99, respectively, by selection for growth on glycerol mini-

mal plates containing 10 mM α-methyl glutamate, a potent inhibitor of growth which enters the cells via the glutamate uptake system (65). This selected against the ability to transport glutamate, and resulted in selection of strains containing lesions in the gltC locus (Essenberg, unpublished results). Figure 2 summarizes the relationships among the various strains in the study.

Genetic Crosses

P1 cml clr 100 phage grown on the desired donor strains were used for transductions. Procedures given in Miller (66) were used for transductions and conjugations and growth.

Growth Curves

Cells were grown in glycerol minimal medium with shaking at 37°C. for approximately 12 hours and diluted 1:100 into tubes of either glucose or glucose-6-P. Periodic turbidimetric records were made of the growth of the strains using a Klett colorimeter with a number 42 filter.

Rates of Uptake

Cells from an overnight culture grown on glycerol minimal medium were diluted approximately 1:100 and grown for about 18 hours. The cells were centrifuged, washed once and resuspended in 140 mM KCl containing 1 mM PIPES, pH 6.6. {^{14}C}-Glucose-6-P was used to measure the rate of glucose-6-P uptake using a filtration assay as reported by Essenberg and Kornberg (49). A volume of 0.095 mls of cells, and 0.05 mls of 2 mM glucose-6-P of specific activity of 2 uC/mmole were added

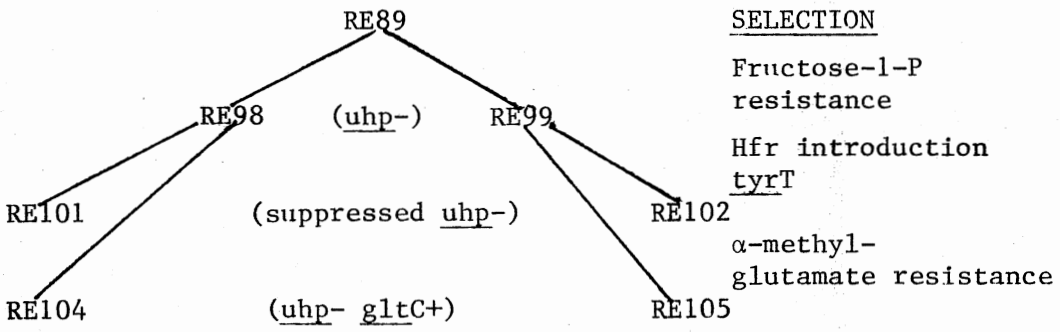

Figure 2. Relationships Between Strains

at 20°C. 0.2 ml samples were removed with an Eppendorf pipette at 10 second intervals over 40 seconds. The cells were collected on a filter of 0.045 um pore size and washed with 2 ml of 140 mM KCl containing 1 mM PIPES. The {^{14}C}-glucose-6-P contained in the cells was counted after drying, using 15 ml of scintillation fluid consisting of 4 grams of 2,5-diphenyloxazole, and 0.2 grams of bis-o-(methylstyryl)-benzene per liter of toluene. The {^{14}C}-content was measured, with less than 2% counting error, on a Beckman LS3150 Liquid Scintillation Counter. A straight line was fitted to the initial 30 second portion of the plots by a least squares method. The values of the slopes of the lines for the rates of uptake had at most 2% standard error of estimate.

Membrane Isolation

Cells were typically grown for 24 hours on 100-200 ml of glycerol minimal medium. This culture was started from an overnight culture grown in 5 ml of LB medium. The cells were harvested, washed, broken, and extracted according to the method of Schnaitman (56). Figure 3 gives the details of this procedure. To establish that the inner and outer membranes were well separated, assays of marker activities known to be located in one of the membranes were used (see Table II). The sugar 2-keto-3-deoxyoctulonate, is located in the outer membrane (55). KDO was measured spectrophotometrically by the method of Warren (67). A modification of the method involving extraction of the chromophore in acidified butanol according to Hammond and Ppermaster (68) was used. CDP-diglyceride:sn-glycerol-3-phosphate phosphatidyl transferase is localized in the inner membrane (69). This enzyme was measured by CDP-diglyceride-dependent incorporation of sn-glycerol-3-phosphate into

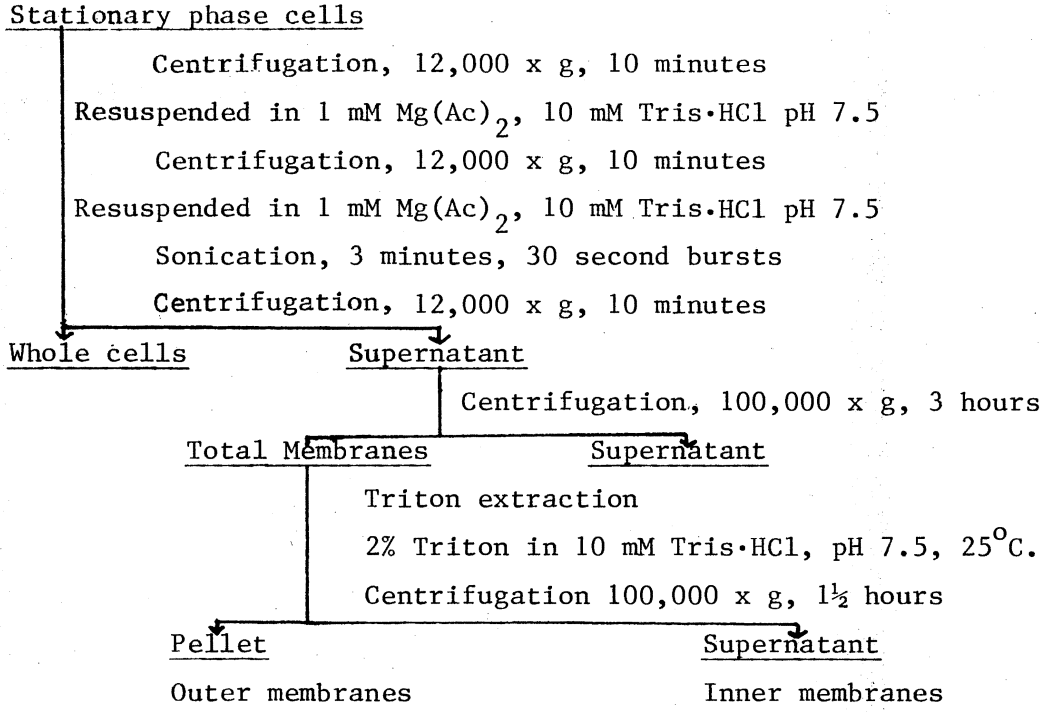

Figure 3. Membrane Isolation Procedure

TABLE II

INNER AND OUTER MEMBRANE MARKER ACTIVITIES

	Protein[1] mg/ml	KDO[2] nmol/mg protein	Sugar[3] nmol/mg protein	PGP Synthetase Activity[4] umol α-gp/mg protein
Triton soluble	1.1	n.d.[5]	16.6	610.9
Triton insoluble	1.5	11.3	272.7	24.0

[1] Using BSA as a standard

[2] Using NANA as a standard

[3] Using galactose as a standard

[4] Glycerol-3-phosphate:CDP-diglyceride phosphatidyl transferase activity

[5] Not detectable

lipid-extractable material (70). Most membrane-bound sugar is localized in the outer membrane (71). Sugar was measured by the method of Dubois et al. (72). Protein content of samples was measured by the method of Lowry et al. (73). Protein content of samples solubilized in Triton X-100 was determined by a modification of this method using SDS to prevent precipitation (74).

SDS Polyacrylamide Gel Electrophoresis

Amberlite XAD-2 beads were used to remove Triton X-100 from inner membrane proteins. The beads were prepared according to the procedure of Holloway (58). The beads were then used to remove Triton X-100 from inner membrane mixtures containing 1-5 mg/ml of membrane protein. The length of time that the proteins were in contact with the beads was either 2 hours at 25°C. or overnight at 4°C. When the samples required concentration they were evaporated to dryness by lyophilization and resuspended in distilled water. Two series of thawing and refreezing could be tolerated witout the aggregation which occurs upon freezing interfering with the electrophoresis. All samples to be electrophoresed were resuspended in equal parts of water and a solution of 10% (v/v) glycerol, 10% (v/v) mercaptoethanol, and 10% (w/v) SDS. The final protein concentration was 10-20 mg/ml depending on the sample. 100-200 ug of protein thus resuspended was heated in a boiling water bath for 5 minutes. SDS gel electrophoresis was performed according to the procedure of Laemmli (75) using slab gel electrophoresis apparatus (76). Essential instructions for preparation and handling of gels were followed (77). Gels contained 5% (w/v) acrylamide in the stacking gel and 10% (w/v) acrylamide in the running gel. Gels were run at 100 volts,

constant voltage, until a current of 15 millamps was reached after which the run was continued at a constant current of 15 milliamps. The run was continued until the dye band had progressed about 10 cm into the 12 cm x 12 cm gel. Gels were soaked after electrophoresis in 10% (w/v) trichloroacetic acid for 10 minutes and then stained overnight in a solution of 5% (v/v) methanol, 7.5% (v/v) acetic acid, saturated in Coomassie Blue R. Gels were destained in a solution of 7.5% (v/v) acetic acid and 5% (v/v) methanol. The quality and state of the reagents used in this procedure needs to be emphasized. The quality of gel obtained depended on the purity of the reagents, particularily the persulfate. Normally it should be light and falky when removed from refrigerated storage in a dark bottle. Partially decomposed (hydrated) persulfate is lumpy and resulted in lack of separation of protein bands as well as very fragile gels which were difficult to handle through staining and destaining procedures.

Flow Dialysis

Flow dialysis was used to measure the binding of {^{14}C}-glucose-6-P to membrane proteins extracted in Triton X-100. The apparatus was described by Klapper (59) and the method was described by Frey (78). The method involved a constant flow of 2 ml/minute of buffer under the dialysis membrane which had been prepared according to the procedure of Littauer (79). The buffer used was the same one that the membranes were solubilized in:10 mM Tris pH 7.5, containing 2% Triton (v/v). A minimum volume under the membrane was present with only entrance and exit holes in the base through which buffer flowed. The top half of the cell had a one cm square hole in it and was screwed to the bottom

plate in each corner. A paraffin gasket of the same dimensions as the top portion of the apparatus was placed between the top and bottom. The dialysis membrane was placed between the gasket and the top portion of the apparatus. Buffer and protein were placed in the top chamber and stirred constantly. Additions were make to the upper chamber and the rate of {^{14}C}-glucose-6-P appearing in the effluent after equilibration was measured. Steady state levels of {^{14}C}-glucose-6-P appeared in the effluent in less than 0.5 minutes. A sample was collected between 2½ and 3 minutes after each addition. 0.2 ml of this sample was withdrawn and counted in 10 mls of scintillation fluid consisting of 40:60 (v/v) 95% ethanol:toluene containing 5 grams of 2,5-diphenyloxazole, and 0.5 grams of bis-o-(methylstyryl)-benzene per liter. Counting was done on a Beckman LS3150 Liquid Scintillation Counter.

Calculations were perfomed according to Frey (78). Background samples (BKG) of effluent were taken after addition of protein and before addition of {^{14}C}-glucose-6-P. Samples taken in steady state after the addition of {^{14}C}-glucose-6-P were designated SSI. The labeled glucose-6-P was released from binding proteins by addition of an excess (100mM) of non-labeled glucose-6-P. The amount of glucose-6-P detected after reaching steady state in this sample (SSF) would be the sum of free and released glucose-6-P after having been bound to the protein. Free glucose-6-P is expressed as G6PF, and ν is the amount of glucose-6-P bound in mmoles/mg membrane protein. The total amount of glucose-6-P added is designated AG6P. P is the protein concentration in mg/ml. Standard (STD) indicates the number of counts per minute obtained per mmole of a known amount of the {^{14}C}-glucose-6-P. This value was corrected by

a dilution factor of 750 in going from the sample chamber to the eluent. (This factor was obtained by adding known amounts of glucose-6-P and measuring the amount of glucose-6-P in the eluent.) Calculations were as follows.

$$G6PF = (SSI - BKG)5 \; / \; STD$$

5 is the factor needed to convert the value to counts per ml of sample.

$$\nu = ((1.2*SSF-SSI)/SSF*1.2)(AG6P/P)$$

In the preceding equation 1.2 is the correction factor for the increase in the volume due to the addition of excess glucose-6-P.

CHAPTER IV

RESULTS

Selection of Mutants

RE89 was chosen as the parental strain from which amber mutants would be selected. Two amber markers were necessary to show simultaneous suppression of the two known amber lesions simultaneous with that of uhp as a result of a single event: the introduction of a suppressor. The uhp^c characteristic was required for the selection by resistance to fructose-1-P and was advantageous as the mutants did not require induction for the various comparisons. RE89 was plated on glycerol minimal plates containing fructose-1-P. In cells with a functioning uhp system, fructose-1-P will enter the cell via the constitutive uhp system and build up internally because of the lesion in fpk. The internal build-up of fructose-1-P will stop cellular metabolism (45). Thus, only cells which cannot transport fructose-1-P will survive this selection. Colonies appearing were compared for growth on glucose and glucose-6-P on plates. Colonies able to grow on glucose and unable to grow on glucose-6-P were chosen. The suppressed version of the mutants (made by matings with a suppressor-carrying strain, H12r8a) grew on glucose-6-P plates. These suppressed strains were cross-streaked with a T4-phage which only causes lysis on cells carrying an amber suppressor to confirm the presence of the suppressor.

Growth and Uptake Comparisons

The mutants were compared in growth properties on glucose and glucose-6-P by growth curves in liquid medium. These showed (Table III) that there was near normal growth on glucose-6-P as compared to glucose in all strains. This is twice as long as might be normally expected from other strains. This was at best an unexpected result. The strains showed significant reduction in actual uptake of glucose-6-P when compared to the parent, and restoration of activity was seen in the suppressed strains in uptake assays. Table IV shows this.

Preliminary Mapping

The mutant strains, RE98 and RE99, were transduced with P1 bacteriophage made from HfrH to verify that the uhp lesions were 50-90% cotransducible with gltC. The data in Table V indicate these loci are cotransducible with gltC. To further prove this point, and to demonstrate that the negative genotype is still present in the suppressed strains, RE101 and RE102 were used as donors in transductions with HfrH. Again, the results shown in Table V demonstrate that the uhp lesions are present in these strains and that they are cotransducible with gltC.

Fine Structure Mapping

The lesions in the two amber mutants were mapped with respect to two previously characterized uhp- mutants and their gltC^{c} derived relatives. Reciprocal crosses of the two amber mutants and the two other uhp mutants showed the lesions to very close to one another. The ratio of uhp+ colonies to gltC^{c} colonies resulting from these crosses of two

TABLE III

GROWTH RATES

Strain	Doubling Time (Hours)	
	Glucose	Glucose-6-P
RE89	1.4	1.2
RE98	1.5	1.3
RE99	1.4	1.5
RE101	1.5	1.0
RE102	1.4	3.0

TABLE IV

INITIAL RATES OF UPTAKE
OF GLUCOSE-6-P

Strain	Rate of Uptake[1]
RE89	5.34
RE98	0.18
RE99	1.56
RE101	13.8
RE102	6.18

[1] nmoles glucose-6-P/mg dry weight/ minute

TABLE V

COTRANSDUCTION WITH *GLTC*

Donor	Recipient	Ratio[1]	% Cotransduction
HfrH	RE98	33/75	44.0
HfrH	RE99	59/196	30.1
RE101	HfrH	45/78	57.6
RE102	HfrH	69/156	44.2

[1]For crosses with HfrH as the donor, the ratio indicates *gltC*^{c} per total glucose-6-P+ selected. For crosses with HfrH as the recipient, the ratio indicates glucose-6-P+ per total *gltC*^{c} selected.

negative mutants should be the probability of recombination between the two lesions to give a positive phenotype. These frequencies are shown in Table VI.

SDS-Polyacrylamide Gel Electrophoresis

Proteins from membranes prepared in this work by the method of Schnaitman (56) when separated by SDS gel electrophoresis give the patterns shown in Figures 4 and 5.

The outer membrane profiles (Figure 4) of RE89, RE98, and RE99 contained the major outer membrane proteins. (Molecular weights were calculated using a plot such as in Figure 6.) In a published report of outer membrane profiles, Schnaitman (56) shows profiles similar to these.

Inner membranes of the strains in this study are very similar to those published (see Figure 5). Groupings of dominant bands in the range of 20-35,000 (4-6 bands) and in the range of 55-70,000 (a triplet and a singlet) agree well with findings in other studies (56, 77, 80-82). Higher molecular weight proteins were commonly seen in profiles of the uhp amber mutants and related strains. Published patterns did not show these. Villarejo (17) found that 10% of the total β-galactosidase in the cell is found to co-purify with the membrane. It is not clear what the function or origin of the high molecular weight proteins is in these strains.

A specific difference of one band which is missing from RE98 and RE99 and is present in RE89, RE101, and RE102, indicates that the protein corresponding to this band is a component in the uhp system.

TABLE VI

RECIPROCAL CROSSES

Donor	Recipient	uhp+/Total	%	χ^2
RE98	RE21	3/425	0.7	1.9 (20%>P>10%)
RE74	RE104	0/328	0.0	
RE98	RE37	2/435	0.4	10.1 (1%>P>0.1%)
RE76	RE104	8/286	2.7	
RE99	RE21	2/337	0.6	0.6 (P>30%)
RE74	RE105	5/428	1.1	
RE99	RE37	2/432	0.4	1.0 (P≅30%)
RE76	RE105	0/234	0.0	

In each cross *gltC*^{c} was selected, and recombinants were tested for glucose-6-P+. % is the percentage of glucose-6-P+/Total. P values are indicated for each χ^2 value given (83).

Figure 4. SDS- Polyacrylamide Gel Electrophoresis of Outer Membranes of uhp Mutants. Molecular weight standards are indicated as (MW x 10^{-3}). Left to right the strains are RE99, RE89, and RE98, with the standards shown to tne right.

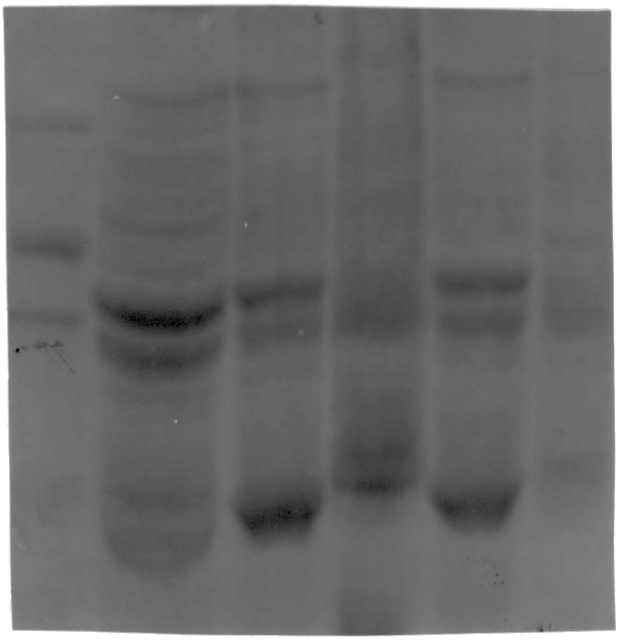

Figure 5. SDS-Polyacrylamide Gel Electrophoresis of Inner Membranes of uhp Mutants. Notations are the same as in the previous figure. Strains (left to right) are RE102, RE99, RE89, RE98, RE101, and molecular weight standards.

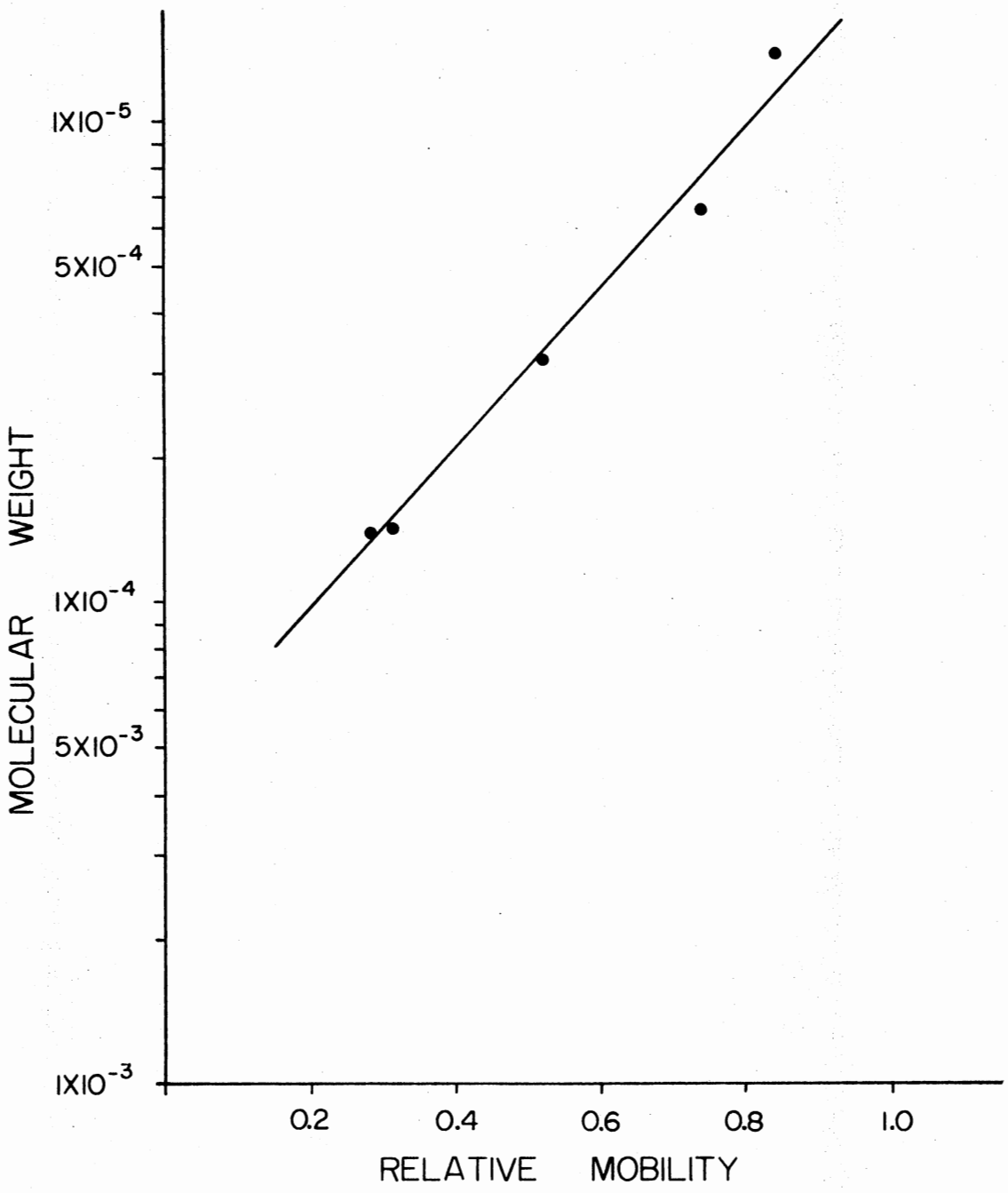

Figure 6. Molecular Weight Calibration Curve

Binding

Binding was first detected by equilibrium dialysis. Flow dialysis was tried to see if more consistent results could be obtained. The binding measurements reported were obtained by flow dialysis, as described elsewhere. The first step in characterizing the binding was to determine the ν_{max} and K_{dis} of the system. The measurements were obtained using different glucose-6-P and protein concentrations (Figure 7). The ν_{max} is 27umole/mg membrane protein. The K_{dis} is 50 um. The latter value is reasonably close to the reported value of the Km of 28 uM for the transport system in whole cells (36).

At this point the specificity of the binding was determined to ascertain if the binding being measured was due to the uhp system or was the result of non-specific binding present in the solubilized membranes. Table VII shows the results with the various compounds which are substrates for the system (fructose-6-P, fructose-1-P, and glucose-1-P) and some conditions which may be expected to release the glucose-6-P, and the combination of glucose and phosphate, which might imitate the structure of glucose-6-P, and some compounds or conditions which should not show any effect on the system.

The effect of Hg++ (a known inhibitor of the system in whole cells) (Essenberg, R. C., unpublished results) was examined. Table VIII shows the effect of different concentrations of glucose-6-P added before and after the additions of different concentrations of Hg++ . The general trend seems to be reduction in binding when the glucose-6-P is added at the same time as the Hg++. If the Hg++ was added prior to the addition of glucose-6-P, no inhibition was seen and an increase was

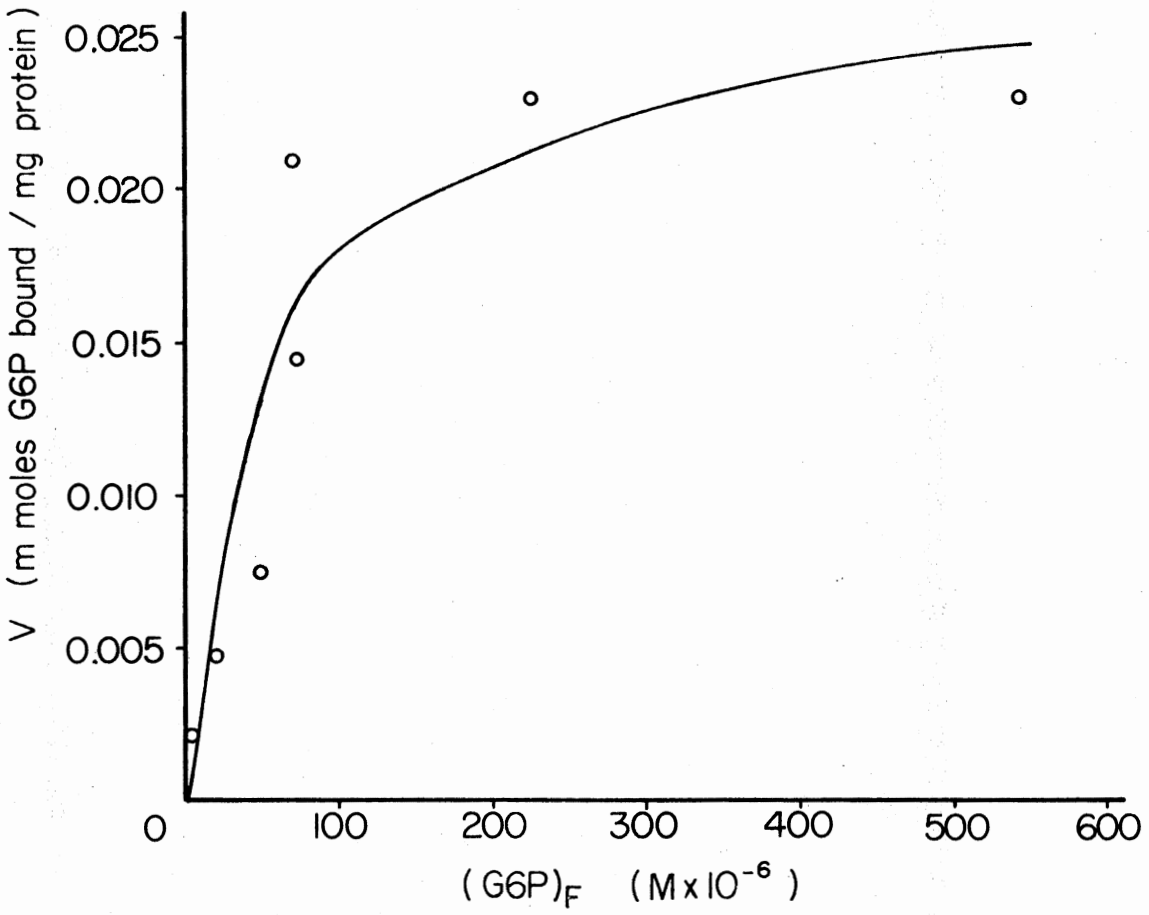

Figure 7. Saturation Curve of Binding

TABLE VII

COMPETITION OF BINDING

Compound (mM) or Condition	% Release[1]
Glucose-6-P (100)	100
Fructose-1-P (50)	86
Glucose-1-P (50)	92
Fructose-6-P (100)	98
Glucose + Phosphate (100)	93
Galactose (100)	0
Citrate (100)	75
KCl (350)	0
EDTA (100)	0
Phosphate (100)	10
pH 6.6	0
SDS (0.1% w/v)	10

[1]% Release represents the fraction of the amount of glucose-6-P (2 mM added concentration added initially) which is released by the test compound compared to that released by 100 mM glucose-6-P.

TABLE VIII

EFFECT OF HG++ UPON BINDING

Hg++(mM)	Time of Addition	Glucose-6-P(mM)	% of Control
10	Before	2	127
10	After	2	91
1	Before	2	102
1	After	2	48
1	After	10	76

Hg++ was introduced either 3 minutes before or immediately after glucose-6-P addition. Control was the amount bound at the specified concentration of glucose-6-P, with no Hg++ present.

observed in one case. Mercaptoethanol was used to attempt to convert the sulfhydryls to the reduced form. Its effect was variable (Table IX). The binding ability of membranes from RE89, RE98, and RE99 was compared and appeared to be similar in all of the strains (Table X). The membranes from uninduced and induced strain 7 and a constitutive derivative were compared in binding capacity. The level of binding in solubilized membranes was compared to the level of uptake seen in whole cells of the same strains (Table XI). The binding ability in the membranes correlates well with the uptake measured in whole cells (Table XI).

To further determine the nature of the binding, trypsin and phospholipase were examined for effect on binding. Trypsin at 5 ug/ml reduced binding by 35% in a 1 minute time period and maintained that level in a 4 minute incubation. Phospholipase had only a slight effect; binding was reduced about 10%. In samples with Triton X-100 removed (as described for SDS gel electrophosresis) the binding was observed at the same level as in samples with Triton X-100 present.

Another interesting fact was that samples of membranes which had been frozen and thawed repeatedly over a period of 3 months, lost no activity. Data for this are shown in Table XII.

TABLE IX

EFFECT OF MERCAPTOETHANOL UPON BINDING

Mercaptoethanol (mM)	% of Control
0	100
10	156
25	102
50	168

Mercaptoethanol was added just prior to the addition of the 2 mM labeled glucose-6-P. The control had no mercaptoethanol added.

TABLE X

COMPARISONS OF AMBER MUTANTS

Strain	ν
RE89	0.0115
RE98	0.0079
RE99	0.0130

Comparisons of ν (mmoles glucose-6-P bound/mg membrane protein) at 2 mM added glucose-6-P.

TABLE XI

COMPARISON OF DERIVATIVES OF STRAIN 7

State	Rate of Uptake[1]	ν[2]
Uninduced	1.08	0.0047
Induced	not measured	0.0110
Constitutive	3.54	0.0125

[1] nmoles glucose-6-P/mg dry weight/minute in whole cells

[2] mmoles glucose-6-P bound/mg membrane protein

TABLE XII

EFFECT OF AGE ON BINDING

Age	Glucose-6-P (mM) Added	ν[1]
Old	2	0.0160
New	2	0.0150
Old	10	0.0700
New	10	0.0700

The ability of frozen membranes to retain the binding ability was measured using a batch of 3 month old membranes which had been repeatedly frozen and thawed, and a freshly prepared batch which had never been frozen.

[1]The value of ν is shown in mmoles glucose-6-P bound/mg membrane protein.

CHAPTER V

DISCUSSION

The goals set forth for the project have been achieved. Amber mutants for the uhp system have been isolated and characterized. The location of the lesions is near minute 81 in the same locus as other uhp lesions. Differences have been found in membrane proteins of amber mutants and control strains, and a component of the uhp system has been tentatively identificed. Binding activity of hexose phosphates has been measured in solubilized membranes under various conditions. The level of binding was too high when compared with published levels of other similar systems.

Growth and Uptake Comparisons

The uhp activity of the amber mutants and control strains was compared by growth on plates, in liquid media, and by measuring uptake of labeled glucose-6-P. All strains grew in a similar manner on plates and in liquid media on glucose. The parent and suppressed amber mutants grew on glucose-6-P, but the amber mutants did not grow when tested on glucose-6-P plates. Uptake measurements showed the mutants did not take up glucose-6-P. The suppressed strains and RE89 took up glucose-6-P equally well. RE101 actually showed more uptake than RE89. This must mean that the amino acid (tyrosine) which was substituted in the protein by the suppressor must function better in uptake than the amino

acid normally found in that position in the protein.

In liquid media, the mutant and parent strains all grew on glucose-6-P with a similar mean generation time, which was slower than is typical in other uhp+ strains. RE102 grew slower than RE99 or RE89. This would seem to indicate that it was not the mutants which were growing, but some altered strain. This change would be possible if the strains were able to compensate for the lack of the uhp component missing in these mutants. Possibilities include induction of another system capable of carrying glucose-6-P; selection for a constitutive or inducible mutant capable of growing on glucose-6-P, originating from the amber mutant in the course of the growth in liquid media; or the possibility that the component lacking in the mutants can be compensated for in growth in liquid media. Liquid cultures were plated before and after the growth. Colonies appearing after the growth on glucose-6-P were smaller than wild type colonies on glucose-6-P, but larger than those of the original negative strain. In the course of transductions, 10^8 cells were plated on selective plates, including glucose-6-P, and no revertant colonies were seen. Low levels of background binding could be expected in the system because of the recognition site at the membrane level, but this should not increase with time. These findings may agree with the uninduced transport level seen by Winkler (36). Winkler's uninduced level is lower than that seen in the mutants. Winkler (36) compared the specificity of the system with that present in uninduced cells. Winkler (36) found that fructose-6-P does not compete with glucose-6-P in the uninduced system but does in the induced state. Also, a difference is noted in fluoride's inhibition in these instances. (Fluoride is used as an inhibitor of the phosphoenolpyruvate-dependent

Roseman system.) Transport as glucose would require a phosphatase to cleave the glucose-6-P. Winkler (36) found inhibitors of the glucose transport system and phosphatase activity had no effect on transport activity in the uninduced state.

The increased lag time seen in growth on glucose-6-P in liquid media in the mutants could be explained if the amber mutations affected a protein which acts as a control element in the system and induction was possible but slow. However, for this to be true, induction would be expected in growth on plates also. A better explanation is possible if the gene altered in the mutants were responsible for energy coupling or translocation in the system. It is possible this factor could be replaced in the liquid situation but not on plates, in correspondence with the growth patterns seen in these strains.

Transductions

Any models of the system must consider that the cotransduction frequencies measured indicate that the region of the genome affected is very small, and that the lesions are located in the same region of the genome as that previously described for uhp mutants (44-48). The two amber mutants were mapped to be within 2.7 map units at the outer limit from two previously described negative mutants, RE21 and RE37 (48). RE99 and possibly RE98 lie between RE37 and RE21. Location of these mutants provides further evidence that a protein producing gene is present on the gltC side of the constitutive mutant mapped by Essenberg and Kornberg (48).

The fine structure map of the region (Figure 8) was constructed using the recombination frequencies shown in Table VI. Distances

between mutants were taken from the recombination frequencies. It had been hoped that the χ^2 values could be used to determine the relative order of the lesions in the 4 strains, RE21, RE37, RE98, and RE99. The χ^2 values were low in all except one case. The actual map distance is identical between the two lesions being mapped in each half of the reciprocal cross. The observed frequencies should be different. In a bacterial transduction an even number of crossovers must occur between the donor and recipient DNA. See Figure 9. In portion A of Figure 9, it can be seen that to obtain a uhp+ from crossing two uhp- strains, (remembering that a crossover must occur on the distal side of gltC because strains carrying gltC^c were selected) four crossovers will be necessary if the locus of the donor is nearer gltC than is that of the recipient. Section B shows that in the case in which the donor's lesion is farther from gltC, only two crossovers are required. In each case the minimum number of crossovers are shown and could be increased by multiples of two. For this reason in a pair of reciprocal crosses, the smaller of the two frequencies should arise from the case of the donor carrying the uhp- lesion being nearer gltC. These considerations are the basis of the ordering of the lesions in the pair RE37 and RE98. RE37 is closer to gltC than is RE98. Other crosses did not show a statistically significant difference in frequency. The fact that the other χ^2 values were low must mean that significant differences in the observed ratios might be seen if larger samples were used, or that crossovers occur in this region with a larger frequency than is normal. High negative interference often occurs when mapping two closely linked markers. This results in groups of crossovers becoming more frequent in a given region of the genome. For the recombination frequencies,

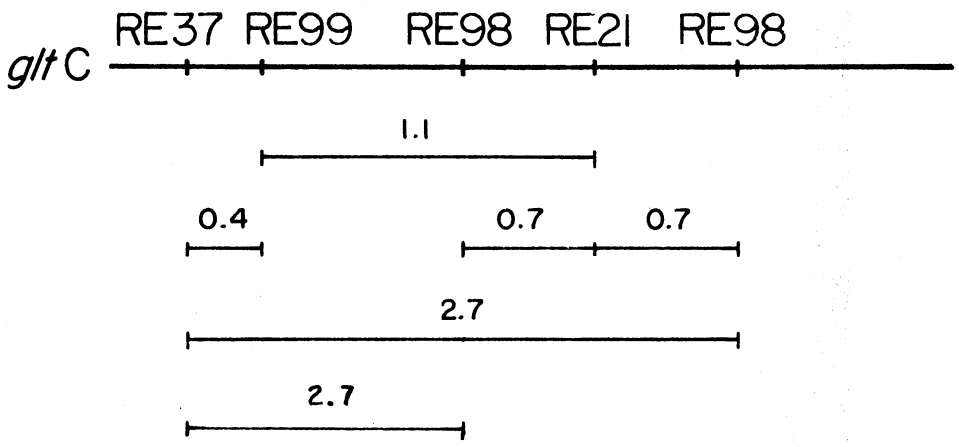

Figure 8. Fine Structure Map

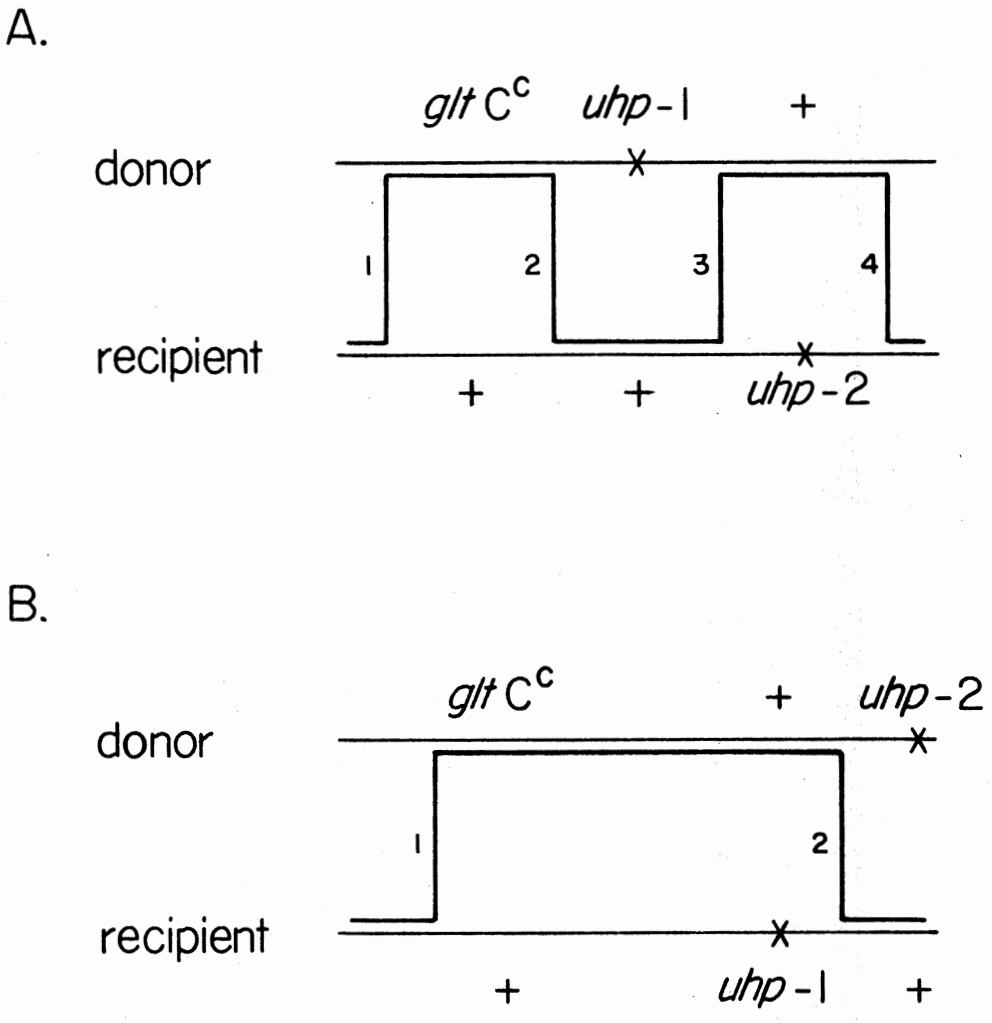

Figure 9. Means of Crossover Occurance

the distances were used to locate RE99 which was 0.4 units from RE37 and 1.1 units from RE21, and was placed between RE21 and RE37. There are two possible locations for RE98 shown in Figure 8 because the order information is lacking with respect to RE21.

Electrophoresis

The electrophoretic band consistently missing in inner membrane preparations in the amber mutants and present in the parent and suppressed strains has an apparent molecular weight of 20,000. Because of the lack of good resolution of molecular weights below this, fragments synthesized in the mutants will not be seen in gels of this composition. The difference does appear to be confined to the inner membrane. If multiple differences actually exist in the protein patterns the differences are most likely to be due to slight variations in the amount of protein placed on the gel.

Some colicins (proteins which act to kill E. coli) act to uncouple energy and active transport in whole cells or membrane vesicles and generally increase permeability to ions, and thus act at the membrane level. Knepper and Lusk (84) have reported that Colicin K caused a disappearance of several membrane proteins, including a protein which has the same apparent molecular weight as that shown for the component missing in the amber mutants. If a component needed for transport of hexose phosphates is also needed for control of ion permeability, a pleitropic effect might be seen if the protein were not present. On the other hand, if another component could be substituted under some conditions, the system might function at near normal capacity.

Because there are many functions which require membrane components and a relatively small space in the membrane to put them, membrane proteins may have multiple functions. An example of this is the lambda phage receptor. In is located in the outer membrane, and has an apparent molecular weight of 55,000. It is also involved in maltose transport (85). The protein apparently aids in transport of maltose by increasing the maltose concentration in the periplasmic space, and allows the transport protein in the inner membrane to sense a higher concentration of maltose, resulting in a higher rate of uptake. This lambda receptor could act as a channel through which maltose is able to move. Outer membrane proteins which function as channels have been reported (53,54).

Spencer and Guest (81,82) used amber mutants to locate the membrane proteins fumarate reductase and succinate dehydrogenase. Their comparisons of amber mutants and wild type strains show that there was only one band lost. In amber mutants used by Boos (12) to examine glycerol phosphate transport, only a reduction in the amount of protein produced was observed.

Two membrane-bound components of a dicarboxylate transport system have been isolated by use of an aspartate-coupled affinity column (86). The proteins have been characterized and examined in a purified state and their binding compared to that in membrane vesicles showing similar results.

A mutant defective in transport of argininine and ornithine has been characterized which has normal periplasmic binding proteins (87). It has been suggested that a regulatory gene affecting the amount of carrier present in the membrane has been altered in these mutants. By

analogy, the uhp mutants described in this work could be lacking a regulatory component similar to the one suggested to be missing in the argininine and ornithine system.

The complexity of interactions of membrane components involved in chemotaxis (movement in response to a chemical), which is in some cases coupled to transport, has been reviewed (88). It should be noted that hexose phosphates do not evoke a chemotactic response in E. coli. Similar problems of looking for proteins which do not have an assay are encountered in the chemotactic system (89). A number of gene products involved in chemotaxis have been identified. By use of membranes isolated from cells treated to synthesize only proteins of genes related to chemotaxis, these genes were amplified. Electrophoresis of these membranes has provided the means of identification of the proteins involved in chemotaxis. Gene products have been identified at molecular weights of 31,000, 39,000, 76,000, 66,000, and 12,000. The genetic region corresponding to the latter three peptides is not large enough to accomodate synthesis of three contiguous genes. To use the same DNA to obtain several proteins, some modification of normal synthesis is necessary. Possibilities include modifications of the reading frame, as in the case of the phate ϕX174 (90), or post-translational modification as has been suggested in the histidine periplasmic binding protein (13) (i.e. the 76,000 molecular weight could be a precursor of the 66,000 and the 12,000 molecular weight proteins.)

More mutants obtained by various methods should be examined to better determine which of these or other possibilities is most likely in the uhp system. The eventual isolation of the protein components from several types of mutants, and structural studies on the proteins

may give insight into the mechanism of transport and of the assembly into the membrane of the components involved in the uhp system.

Binding

The binding parameters have been compared to published values for the lactose system and that of the uhp system. The apparent K_{dis} observed is reasonable, but the v_{max} of the lactose system is 2.3 nmoles/mg protein (91). There is too much binding present to be accounted for by the amount of protein present. The nature of the binding is not clear from the experiments reported in this study. The various substances used to examine the release of bound glucose-6-P showed proper specificity except the combination of glucose and phosphate which is not known to compete with glucose-6-P in whole cells, and citrate, which has no effect on the transport system in whole cells (Essenberg, R. C. unpublished results). Attempts to raise the ratio of bound glucose-6-P to that of potential inhibitors were not successful, indicating the proper ratios were not used (i.e. the sum of inhibitor plus labeled glucose-6-P concentrations should be 5 mM). The action of SDS which does not release all of the glucose-6-P in the course of the experiment, must imply that unfolding the protein by SDS does not release the bound glucose-6-P. Possibilities include that the glucose-6-P is bound in such a way that unfolding of the protein does not cause release, SDS does not unfold the protein, or the glucose-6-P is not bound solely to protein. The lack of full release by trypsin may substantiate the latter possibility. Also, since the binding was not released by the phospholipase, this could imply that a combination of protein, with lipid, and/or carbohydrate provided the structure of

the binding site, and that only a specific interaction with glucose-6-P will cause complete release.

Because of the possible role of sulfhydryl regents on the uptake system (49) and the possible role of sulfhydryls in cycling of the transport process (92) the effect of mercaptoethanol and Hg++ were examined. Hg++ apparently has different effects depending on the incubation conditions (i.e. whether glucose-6-P is added at the same time or after Hg++). The effect of Hg++ may be to cause a conformation change to increase binding if glucose-6-P is added after Hg++. The effect of the Hg++ when added with the glucose-6-P may be to block binding or cause release of glucose-6-P when it is added together. The effect of mercaptoethanol is not apparent at this point.

The binding capacity seen in strains which were induced, uninduced and constitutive for the uhp system (all derived from strain 7) showed the proper relationship between binding and the genetic state of the uptake system. This also correlates well with the relative uptake seen in the strains in whole cells.

In comparisons of the amber mutants with the parent strain, one showed 70% and the other 116% of the binding level seen in RE89. This might indicate that the mutation was not in the component of the system which was used for binding. (The evidence of the mapping points to the fact that the protein must be synthesized as a part of the uhp system, and thus this must indicate that there is a component not involved in binding required for activity, if the binding observed is due to the uhp system.)

The K_{dis} and the specificity of the binding activity observed are consistent with that of the uhp system, but the v_{max} was too high (as

compared to values from the lactose system) to be due entirely to the uhp system.

A Possible Model

A model for the system based on the information gained to date seems to require at least two components. One protein (possibly associated with lipid or carbohydrate) which recognizes the glucose-6-P, then requires interaction with another component to accomplish transport. The second component, to be called the coupling component, may be required for energy coupling, induction, or possibly translocation.

The amber mutants would be lacking the coupling component, as described above. Under some conditions in the absence of the coupling component, another component may act as a substitute (as in liquid growth seen in this study).

To examine the model in more detail will require more mutants isolated in a variety of ways. The identification of other components would be helpful. Eventual study of the system in a relatively pure form in vesicles will provide some evidence for the role of the component identified here and the others which are not identified, in the operation of the entire system. Comparisons along the lines followed in this study as to uptake, growth, binding, and electrophoretic patterns of isolated membranes from other mutants should provide insight into the actual mechaism by which the uhp system functions.

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