IDENTIFICATION OF PROTEINS ASSOCIATED WITH *UHP* SYSTEM OF *E. COLI*

By

JOHN PAUL WILDER

Bachelor of Science

McNeese State University

Lake Charles, Louisiana

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Thesis Approved:

Richard C Essenberg
Thesis Adviser

Earl D Mitchell Jr

Ulrich Melcher

Norman N Durham
Dean of the Graduate College

PREFACE

I would like to thank the members of my committee, Dr. Ulrich Melcher, Dr. Earl Mitchell and my adviser, Dr. Richard Essenberg, for their assistance and patience. I would also like to acknowledge the guidance of Dr. Stearns Rodgers of McNeese State University for recommending Oklahoma State University to me. The knowledge and education given to me by the faculty of the Biochemistry Department and the financial assistance granted to me is gratefully acknowledged.

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NOMENCLATURE

A_{680} - absorbance at 680 nanometers

araC - gene for regulatory protein of arabinose transport system

ATP - adenosine triphosphate

BSA - bovine serum albumin

DNA - deoxyribonucleic acid

F1P - fructose-1-phosphate

F6P - fructose-6-phosphate

g - gravity

glu - glucose

gly - glycerol

G6P - glucose-6-phosphate

kd - kilodalton

K X g - 1000 times gravity

LacZ - gene coding for β-galactosidase

M - molar

mA - milliamp

malF - gene coding for cytoplasmic membrane protein of maltose transport system

mg - milligrams

ml - milliliters

mM - millimolar

mmol - millimoles

mV - millivolts

M6P - mannose-6-phosphate

N - normal

nm - nanometers

NDGP - glucosamine-6-phosphate N diethylsulfone

PIPES - piperazine-N-N'bis(2-ethanesulfonic acid)

pyrE - gene coding for orotate phosphoribosyl transferase

SDS - sodium dodecyl sulfate

SDS PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

uhp - gene for uptake of hexose phosphates

uhpR - gene coding for regulatory protein of uhp systems

uhpT - gene coding for transport proteins of uhp system

µCi - microcurie

µl - microliter

µM - micromolar

µm - micrometer

µmol - micromole

v - volume

CHAPTER I

OBJECTIVES

The primary goal of this study was the identification of the protein(s) of the hexose phosphate uptake system of Escherichia coli. The study was carried out using SDS-PAGE and affinity chromatography.

CHAPTER II

INTRODUCTION

The diffusion of molecules across cellular membranes centers around two basic types: simple or non-mediated diffusions and mediated diffusion. Mediated diffusion can be further separated according to energy requirement. If energy is required for transport then transport is considered to be active. If energy is not required then transport is considered to be passive.

Active transport is typified by the hexose phosphate uptake system of Escherichia coli. It requires energy in the form of a H^+ gradient (1, 2). This H^+ gradient can be generated by either a pH differential or an electrochemical potential, interior negative, across the cellular membrane (3-5). This energy requirement follows the chemiosmotic mechanism for energized uptake of hexose phosphates.

Active transport systems in Escherichia coli can further be separated based upon their requirement for a binding protein located in the periplasmic space (6). The systems containing periplasmic binding proteins utilize high energy phosphate bonds as an energy source. The other systems, without periplasmic binding proteins, exemplified by lactose transport system, are coupled to the H^+ gradient.

There are metabolites, however, which are not transported by just a single system. Arabinose is transported by both types of

systems (7). The araE gene system is associated with the nonperiplasmic binding systems (8). This system acts as a sugar-H^+ symport and is powered via H^+ gradient or electrical potential. The other transport system is associated with the araF gene and is thought to be energized by a phosphorylated product of glycolysis (9).

The hexose phosphate uptake system of Escherichia coli has characteristics which make the study of it confusing. Based upon osmotic sensitivity of the uhp system, it appeared to require a periplasmic component (11). However, no such component has been found to date (Essenberg, unpublished, 12). The requirement for energy is the establishment of a membrane potential due to $[H^+]$. This requirement varies according to the pH of the external solution (5). LeBlanc et al. (5) showed that with an internal pH equal to 7.5 uptake of G6P was due to the electrochemical potential at external pH > 7.5 and the ΔpH at external pH $\simeq$ 5.5. These facts suggest that this system is not simply a nonperiplasmic component system or a periplasmic component system.

Inducibility of the uhp system was studied by Winkler (13), Pogell et al. (14) and Dietz and Heppel (15). Winkler and Pogell et al. showed that glucose-6-phosphate could induce the system while Dietz showed that 2 deoxyglucose-6-phosphate was also an inducer of the system. These are the only known inducers of the system and speculation was that, crossing of the membrane by these compounds was not needed for induction. Evidence for this came from Dietz and Heppel (15). They showed that a mutant unable to utilize glucose-6-phosphate as a metabolite could accumulate glucose-6-

phosphate up to 60 mM if grown on glucose. Even at these high internal concentrations, expression of the uhp gene does not occur unless G6P is added to the external medium.

More conclusive and direct evidence has come from Shattuck-Eidens and Kadner (16). The lacZ gene was fused with the uhpT gene such that the expression of β-galactosidase was under the control of the uhpT gene promotor. Using strains with this fusion, the expression of β-galactosidase was shown to occur in strains unable to transport glucose-6-phosphate. It was also shown that the K_m for induction is much less (1.1 μM G6P) than had previously been thought.

Use of mutant strains has been of invaluable aid in the study of the uhp system. The most current linkage map (17) shows that the uhp gene maps between minute 81 and 82. It is located after pyrE. Selection of uhp^- mutants was accomplished by Kornberg and Smith (18) using acetate as a carbon source. These mutants were devoid of phosphoenolpyruvate carboxylase activity so that the presence of hexose phosphates in the medium stopped growth. Thus, if strains grew on a combination of fructose-1-phosphate (a substrate of transport) and acetate, then they were deemed uhp^-. They were further able to isolate constitutive mutants by selecting those strains capable of growth on fructose-1-phosphate (19). Fructose-1-phosphate has been shown to be a substrate of the transport system but does not act as an inducer (13). Essenberg and Kornberg (19) used ethyl methane sulfonate or nitrous acid mutagenesis in conjunction with penicillin to isolate both uhp^c and uhp^- mutants.

Clarke and Garbon (20) created a colony bank of ColE1 hybrid plasmids representing the entire E. coli genome. Essenberg (unpub-

lished data) has screened this colony bank and found five plasmids capable of correcting a uhp^- strain with a point mutation.

Possible Approaches

Since the uhp system is inducible, protein synthesis is required for transport to occur. With this in mind, SDS-PAGE of membranes of uhp^+, uhp^c and uhp^- mutants should show a difference in the proteins synthesized. The proteins involved in transport would be present in the uhp^c and uhp^+ (induced) mutants but not present in the uhp^- and uhp^+ (uninduced) mutants.

MacPherson et al. (21) used dual labeling of cellular proteins to identify the components associated with the araE transport system. ^3H or ^{14}C labelled amino acid mixtures were added to cultures with arabinose present or absent. Two approaches were used. In one case, ^3H labelled amino acids were used with the arabinose culture and ^{14}C labelled amino acids were added to the uninduced culture. The second case used the reverse situation with ^{14}C labelled amino acids added to the induced culture. They found two proteins, 27 and 36 Kd different between induced and uninduced culture. For further proof that the proteins indicated above are involved in arabinose transport, they labelled membrane proteins with N-ethyl maleimide. N-ethyl maleimide is an irreversible inhibitor of arabinose transport. They identified two proteins induced by arabinose. One, a 27 kilodalton protein, is not lost in mutants incapable of transporting arabinose and one, a 36 kilodalton protein, is lost and binds N-ethyl maleimide irreversibly.

A second technique used by Shuman et al. (22) involves the fusion

of an easily detectable protein with the gene in question. They fused the lacZ gene which codes for β-galactosidase to the malF gene producing a hybrid protein with the malF coded sequence at the NH_2 terminal region. Using antibodies to β-galactosidase the hybrid protein was isolated and used to produce antibodies to the malF gene product. These anti-malF antibodies were then used to isolate the malF gene product. It was found to have a molecular weight of ∿40,000 daltons and is located in the cytoplasmic membrane.

Affinity chromatography is another technique used in the identification of transport components. Wilcox et al. (23) used a sepharose column with 4 aminophenyl-β-D-6-deoxy galactopyranoside attached to isolate the araC gene product. This protein binds to the araDNA showing it is involved in the expression of the ara genes. While this protein is not membrane bound or involved in the transport directly, this experiment shows that the technique is quite useful for isolating proteins.

Present Study

Work with osmotically shocked cells suggested the existence of a periplasmic component involved in the transport of hexose phosphates (11). However, no component has been found to date. It has been shown that isolated membrane vesicles are capable of taking up glucose-6-phosphate if they are supplied with an external energy source such as lactate (5), which strongly suggests no binding proteins is involved.

With these approaches in mind and these facts available, the following approach was attempted. Since evidence had indicated that the components were primarily located in the cytoplasmic membrane,

isolation of inner membranes of uhp^c, uhp^+ (induced and uninduced) and uhp^- strains was performed using Schnaitman's procedure (24). This procedure is simple and relatively fast in separating not only membranes from the cytoplasm but also in separating the cytoplasmic membrane from the outer membrane. The cytoplasmic membrane is soluble in Triton X-100 if Mg^{++} is present but the outer membrane is not. SDS-PAGE of these membrane preparations was then carried out to detect differences in the proteins of the inner membrane. Samples applied to the gels contained the same quantity of protein to preclude any differences based solely on protein quantity.

The second approach is the use of affinity chromatography. Isolated inner membrane preparations were applied to either a batch or column chromatography. The relative binding abilities of proteins could be determined using the column approach, whereas the batch technique is useful in separating large quantities of proteins.

CHAPTER III

METHODS

Strains Used

Strains used in this study are shown in Table I along with their genotype, source and functionality of uhp systems. RE215, RE216 and RE217 are uhp^- on the chromosomes but the plasmids render them uhp^c.

Growth Conditions

Strains were grown in medium A of Davis and Mingioli (25) to a cell density of 0.6 mg/ml as measured by A_{680}. Carbon sources (at 10mM) were glycerol, glucose-6-phosphate, or fructose-1-phosphate for RE92 and RE144. The remaining strains were grown with glucose, glucose-6-phosphate or fructose-1-phosphate (10mM) as the carbon source at 37^o. RE92 and RE144 were grown at 30^o. Testing of uhp^c strains was by positive growth on fructose-1-phosphate while uhp^- strains were tested by lack of growth on glucose-6-phosphate and positive growth on glucose. uhp^+ strain was tested by positive growth on glycerol or glucose-6-phosphate and lack of growth on fructose-1-phosphate.

Uptake on Cells and Spheroplasts

Cells were collected at near mid log phase ($\sim$0.6 mg/ml) and washed twice with 1 mM Pipes, 140 mM KCl (pH 6.6). They were resus-

TABLE I

STRAINS USED

Strain	uhp Functionality	Genotype	Source
RE92	+	fda^{ts}	Lab stock
RE144	c	fda^{ts}, uhp^{c}	Spontaneous mutant of RE92
RE213	-	uhp 40, recA56	Lab stock
RE215	c	pLC40-33/RE213	RE213 X RE162
RE216	c	pLC35-4/RE213	RE213 X RE163
RE217	c	pLC17-47/RE213	RE213 X RE164

pended to a cell density of 0.6 mg/ml in medium A. To 0.9 ml of cells, 0.1 ml of 2 mM G6P (2 μCi ^{14}C G6P/ml) was added.

Spheroplasts were formed according to Witholt et al. (26) and stabilized with 20 mM $MgSO_4$ in 100 mM $NaPO_4$ (pH 6.6) at a cell density of 0.6 mg/ml. To 0.9 ml of spheroplasts, 0.1 ml of 2 mM G6P (2 μCi ^{14}C G6P/ml) was added.

At 30, 60 and 90 second intervals, aliquots (0.1 ml) were collected on membrane filters (0.45 μm pore size), washed with 5 ml of 1 mM Pipes, 140 mM KCl (pH 6.6) in the case of cells and 20 mM $MgSO_4$, 100 mM $NaPO_4$ (pH 6.6) in the case of spheroplasts. The filters were air dried overnight or dried for four hours at 120°. The retained activity was quantitated in toluene scintillant (4 gm of 2,5 diphenyl oxazole and 0.2 gm bis-0-(methylstyrl)benzene in 1 liter of toluene) with a Beckman LS3150T liquid scintillation detector. Efficiency of counting was determined by counting a known sample under the same conditions as the unknown. Conversion of counts per minute/filter to nmoles G6P was performed by using specific activity of G6P as determined by counting a sample.

Uptakes on Vesicles

Vesicles were formed using the methods of Kaback (27), substituting Na^+ for K^+ in all buffers. Vesicles were frozen at -77° until used or used immediately. Uptakes were performed using either K^+-valinomycin or lactate as energy source.

Vesicles were loaded with K^+ by incubating the vesicles in 0.5M KPO_4 (pH 8.0) at 37° for 30 minutes. The procedure of Hirata et al. (28) was used to perform the uptakes except for the following - filters

were washed with 0.28M sucrose, 13 mM $MgSO_4$, 100 mM Tris-maleate (pH 7.0) (TMS buffer) instead of 0.4M sucrose, 10 mM $MgSO_4$. Filters were dealt with as in cells and spheroplasts.

Lactate energized uptakes were performed according to Dietz (29) except that vesicles were washed on filters with the TMS buffer used in K^+-valinomycin uptakes. Filters were dealt with as above.

Affinity Chromatography

Attachment of glucosamine-6-phosphate to Sephadex G-50 was performed according to the procedure of Porath (30) except that Na/BO_4 was used as the linking buffer instead of Na/CO_3. For radiolabelled proteins, columns of 2 ml were used. For non-radiolabelled proteins, either a batch technique or a column with 5 ml of activated wet gel were used. The batch method consisted of incubation of proteins with activated gel at 37° under slow agitation for 30 minutes, followed by centrifugation at ∿2,000 x g and removal of supernatant. The gel was then washed five times with 2 ml of 50 mM Tris-HCl, 50 mM NaCl (pH 7.5) (Buffer T) followed by washing with Buffer T plus 1.0 mM F6P or M6P in the same manner and then washing with Buffer T plus 1.0 mM G6P as before. Supernatants from the same buffers were combined, dialyzed against 10 mM Tris-HCl (pH 7.8), lyophilized and prepared for electrophoresis.

The elution buffers for the columns were the same as those for the batch technique except only Buffer T and Buffer T plus 1.0 mM G6P were used for radioactive proteins. Detection of proteins in fractions was by A_{280} or modified Lowry (nonradiolabelled proteins) and by scintillation counting (radiolabelled proteins) in toluene/ethanol

(60/40 v/v) scintillant. Fractions with nonradiolabelled protein were readied for electrophoresis as in batch method.

Analytical

Determination of proteins were performed using a modified method of Lowry (31) and the method of Bradford (32). BSA was used as the standard protein.

K^+ ions were determined using glass K^+ ion selective probe in conjunction with a Beckman Titrometer. K/PO_4 was used as standard to produce a standard curve. This curve was used to convert mV observed to mM $[K^+]$.

O_2 utilization was determined with a Clark O_2 electrode. To 2.2 ml of TMS buffer, 100 µl of vesicles (0.1 mg/ml) or cells (0.6 mg/ml) was added and O_2 utilization was monitored. At 5 minute intervals, lactate (50 mmol) and NaCN (1 µmol) were added.

Divinyl sulfone-N-glucosamine-6-phosphate was determined according to the procedure of Elson and Morgan (33) and Reissig et al. (34). Glucosamine and N-acetyl-glucosamine were used as the standards.

Analysis of gels for attached glucosamine-6-phosphate was performed on 1-5 mg of gel in 500 µl of deionized water using a modified assay of Chen et al. (35). Cleavage of phosphates from gel matrix was accomplished using 2.0 N KOH at 98^o for 30 minutes. 2.0 N KOH was neutralized with 2.0 N HCl. 1% ascorbic acid and 0.42% $(NH_4)_4$ Mo•$4H_2O$ in 1 N H_2SO_4 produced a characteristic blue color absorbing at 750 nm. For standard, cellulose phosphate resin (1 meq $PO_4^=$/gram of gel) was used.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

was performed according to Laemmli (36). Gels were stained with either coomassie brilliant blue or silver nitrate (37). Slab gels were run at 7 mA overnight, then current was increased to 12 mA until terminated. Gels were either 10% acrylami-e with 4% stacking gels or linear gradients of 8-15% acrylamide with 4% stacking gels.

Gels stained with coomassie brilliant blue were photographed using a red filter and panatomic X (Kodak) film. Gels stained with silver nitrate were photographed with a magenta filter and panatomic X film. The magenta filter reduced the background created by the silver nitrate staining method.

Samples for electrophoresis were prepared as per Laemmli (36).

Membrane Protein Isolation

The procedure of Schnaitman (24) was followed in isolating the inner membrane proteins except that the removal of Triton X-100 was not accomplished with XAD beads but with the chloroform/SDS procedure of Horikawa (38).

Radiolabelling of Membrane Proteins

0.1 ml of a culture of cells at mid-log phase ($\sim$0.6 mg/ml) were added to 9.9 ml of medium A with glycerol (10 mM) as carbon source. 10 µCi/10 ml final solution volume of ^{14}C Leu (for uhp^{c}) or 100 µCi/10 ml ^{3}H Leu (for uhp^{+} (uninduced)) was added and growth continued for $\sim$4 hours.

RESULTS

Cell Selection

As shown in Table II, the strains were true to their uhp classification in Table I. RE92 could grow on glycerol, glucose or glucose-6-phosphate but would not grow on fructose-1-phosphate, indicating that it is wild type inducible strain. RE144, RE215, RE216 and RE217 were all capable of growing on fructose-1-phosphate, indicating that they are constitutive strains. RE213 was unable to grow on either hexose phosphate which is indicative of a negative strain.

Uptake of G6P by Cells

Strains were tested to see if G6P uptake could occur and if the strains were inducible (Table III). RE92 would only take up G6P if it were grown on G6P as carbon source or if G6P were added as inducer. RE213 was incapable of taking up G6P even if G6P were added to growth media in greater than inducing amounts (2.5 mM vs. 1.1 μM). RE144 and RE215 showed uptake of G6P but did not exhibit increased uptake when 2.5 mM G6P was added to the growth medium. RE216 and RE217 took up G6P when grown on glucose and they exhibited increased uptake when 2.5 mM G6P was added to the growth medium.

TABLE II

GROWTH CHARACTERISTICS

Strain	Growth on			
	Gly	Glu	G6P	F1P
RE92	+	+	+	-
RE144	+	+	+	+
RE213	x	+	-	-
RE215	x	+	+	+
RE216	x	+	+	+
RE217	x	+	+	+

x = Not tested.

TABLE III

G6P UPTAKE OF STRAINS

Strain	Uptake Rate (nmol G6P/mg Cells•min)	G6P[a] (2.5mM)	% Increase
RE92	0.178	-	
	0.310[b]	+	70
RE144	24.7	-	
	27.2	+	10
RE213	0.662	-	
	0.548	+	0
RE215	3.60	-	
	3.57	+	0
RE216	10.5	-	
	13.9	+	32
RE217	9.39	-	
	13.2	+	40

[a]Added ∿4 hours prior to cell harvest.

[b]This is lower than expected. Probably due to short induction time.

Uptake of G6P by Spheroplasts

Spheroplasts were tested for their ability to take up G6P in order to further establish that the components of the uhp system are primarily located in the inner membrane. RE144 showed uptake rates in the same order of magnitude but were about one half that of cells. RE92, uninduced prior to spheroplast formation, showed no change in uptake rates between spheroplasts and cells (Table IV). These results imply one or two things have occurred. Either a component of the uhp system is missing and that that component is located in the periplasmic space or outer membrane or the spheroplasts are damaged and unable to energize the uptake of G6P fully.

Spheroplast formation with RE213 and daughter strains could not be accomplished as measured by lysis in deionized water. No explanation for this is given here in this report. This could be the subject of another study. For this reason, no uptake measurements were performed on spheroplasts of these strains.

Uptake of G6P by Vesicles

Here again, the studies were performed only on RE92 and RE144 because of the inability to form spheroplasts with the other strains. This approach had two objectives, one being to obtain further proof that the uhp components are located in the inner membrane and the other to set up further experiments for reconstitution studies.

Using vesicles of RE92 and RE144, two approaches were attempted. Initially, K^+-valinomycin energized uptakes were performed on vesicles. These experiments did not give consistent results, so measurements were performed to determine if the vesicles were actually loaded with

TABLE IV

G6P UPTAKE BY CELLS AND SPHEROPLASTS

Strains	Uptake (nmoles/mg•min)	
	Cells	Spheroplasts
RE92	0.54	0.62
RE144	11.2	5.11

K^+. These measurements showed that the vesicles were not loaded with K^+ as much as expected and that the vesicles leaked out the K^+ they did contain. This explained the inconsistency of the results.

The second approach was the energizing of the vesicles with lactate. Lactate dehydrogenase is present in vesicles (39) and is capable of establishing a H^+ gradient across the vesicle membrane. RE144 vesicles accumulated more G6P than RE92 vesicles and did show an increase in G6P taken up versus time. RE92 did not show uptake of G6P above the initial measurement (Figure 1).

In order to discover the cause for this, the utilization of O_2 by the vesicles was measured (Figure 2). The vesicles were shown not to use O_2 which could explain why the experiments failed. Possible reasons for this lack of O_2 utilization are lactate dehydrogenase was non-functional or the vesicles were not concentrated enough. To test the system, cells were used to measure O_2 utilization. They showed utilization and the cells were sensitive to NaCN.

This approach was abandoned because of these technical difficulties. It had previously been established by Dietz (2) that uptake of G6P could occur in vesicles energized by lactate.

SDS-PAGE

Electrophoresis of the inner membranes was used to test for differences in proteins between the various strains. Comparison (Figure 3) of RE92 (uninduced), RE92 (induced) and RE144 inner membranes revealed three polypeptides which could be attributed to the uhp gene. Their apparent molecular weights are 27,000, 35,000, and 64,000. They are present in RE92 (induced) and RE144 but not in

Figure 1. G6P Uptake on RE92 (● and ■) and RE144 (o and □) Vesicles with (■ and □) and without (● and o) Lactate.

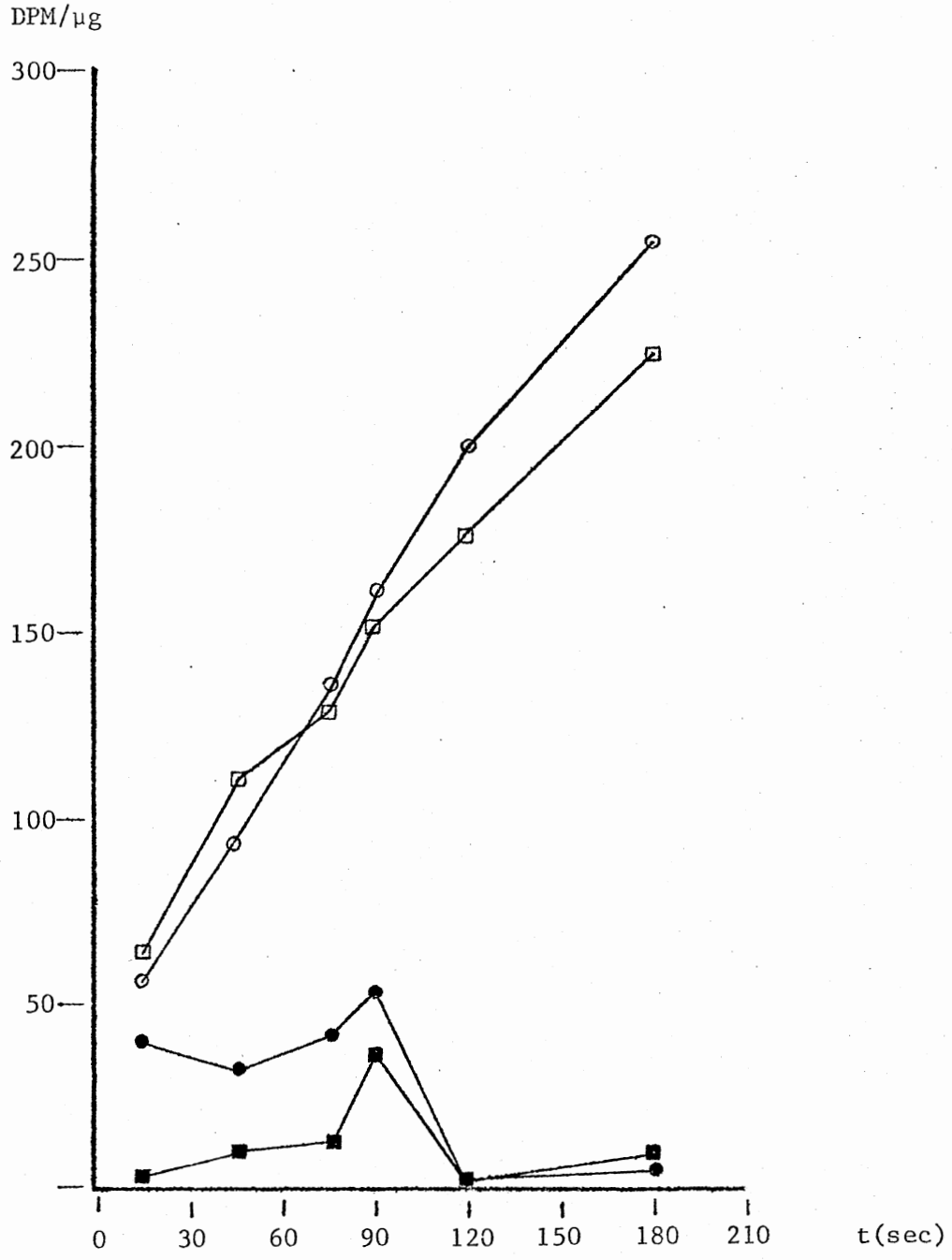

DPM/µg
300
250
200
150
100
50
0
30
60
90
120
150
180
210
t(sec)

Figure 2. O_2 Utilization. (a) RE92 cells; (b) RE144 vesicles; (c) RE92 vesicles.

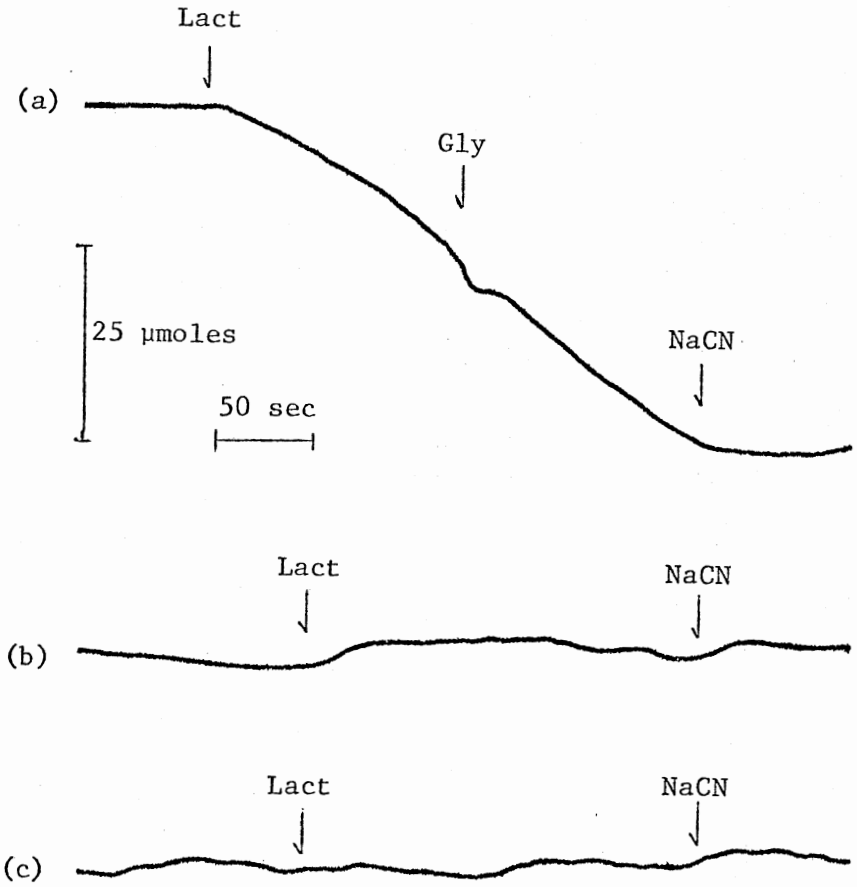
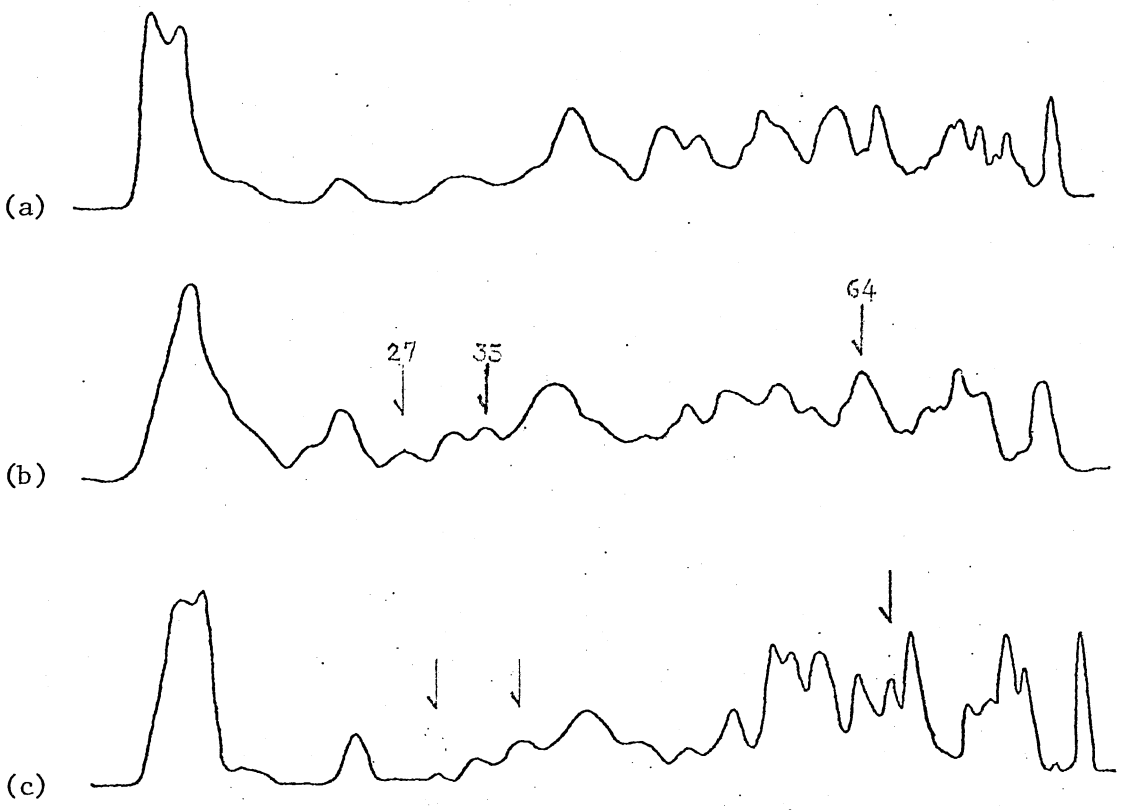

Lact
(a)
Gly
25 µmoles
50 sec
NaCN
Lact
NaCN
(b)
Lact
NaCN
(c)

Figure 3. Density Scan of SDS PAGE of Inner Membranes. (a) RE92 uninduced; (b) RE92 induced; (c) RE144. Arrows indicate differences.


(a)
(b)
27
35
64
(c)

RE92 (uninduced). RE92 (uninduced and induced) contains a polypeptide of molecular weight 22,000 which is not as clearly seen in RE144.

Comparison of inner membrane polypeptides of RE213 with those of its plasmid containing daughters (Figure 4) showed two proteins present in plasmid strains not present in parent strain. Their molecular weights are 37,000 and 70,000. There is a polypeptide of molecular weight 22,000 present to a much greater extent in RE213, RE216, and RE217 than in RE215.

Affinity Chromatography

Attachment of glucosamine-6-phosphate by an N-linkage to Sephadex with divinyl sulfone was accomplished by the procedure of Porath (30). Prior to attachment of glucosamine 6-phosphate-divinyl sulfone (NDGP) to Sephadex, NDGP was tested to see if it was an effective inhibitor of glucose-6-phosphate uptake. As shown in Table V, 39 μM NDGP will inhibit uptake of G6P (40 μM) by ∿ 46%. Using phosphate analysis, it was found that ∿55 μmoles of glucosamine-6-phosphate/gram of dry gel could be attached.

In order to show that the ligand-matrix would retain proteins as desired, radiolabelled proteins were washed through the column. As shown in Figure 5, proteins are in fact retarded and some will elute only with G6P.

Experiments were then designed to isolate and identify membrane proteins which exhibit an affinity for G6P. Affinity chromatography in conjunction with SDS-PAGE was used to isolate and identify these proteins. Column chromatography showed that the uhp^c strains RE144,

Figure 4. Density Scan of SDS PAGE of Inner Membranes. (a) RE217; (b) RE216; (c) RE215; (d) RE213. Arrows indicate differences.

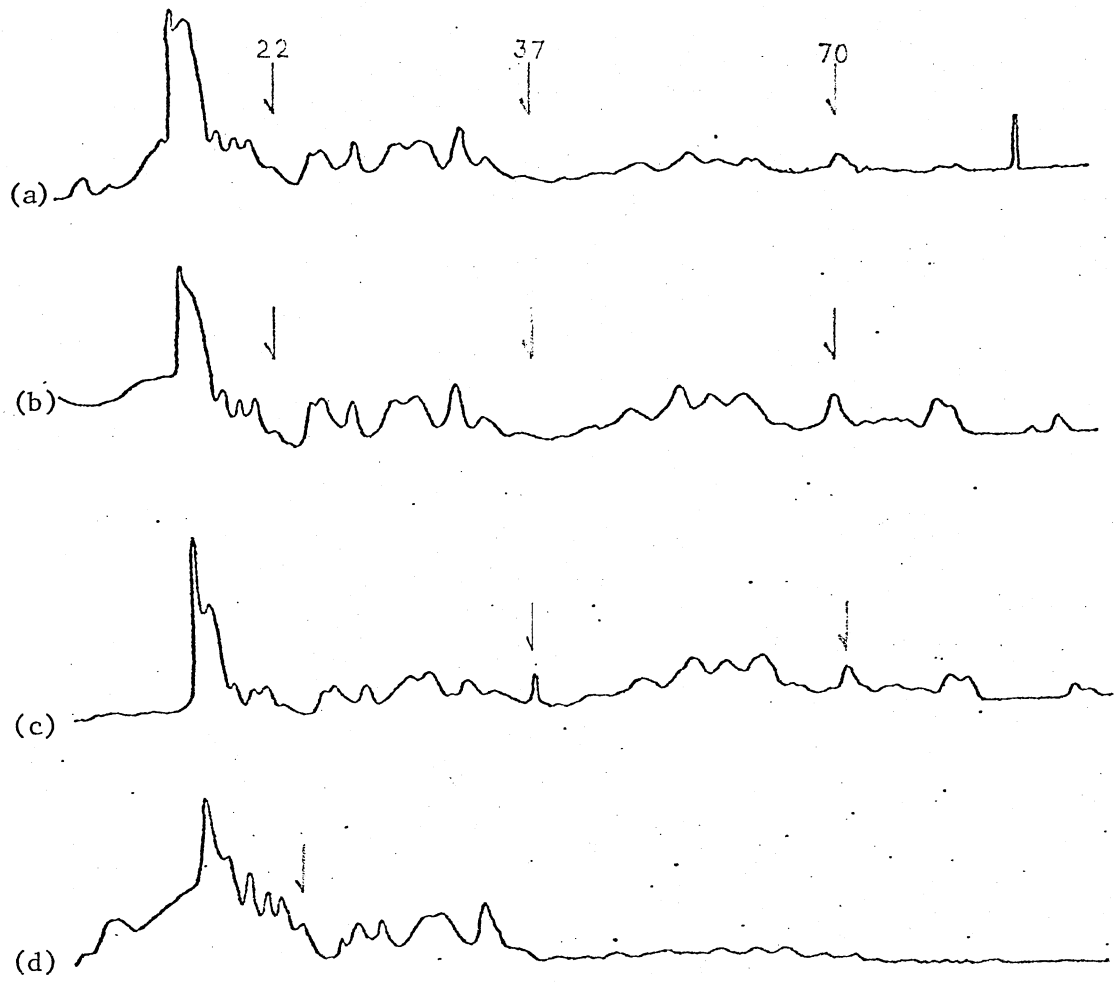

22
37
70
(a)
(b)
(c)
(d)

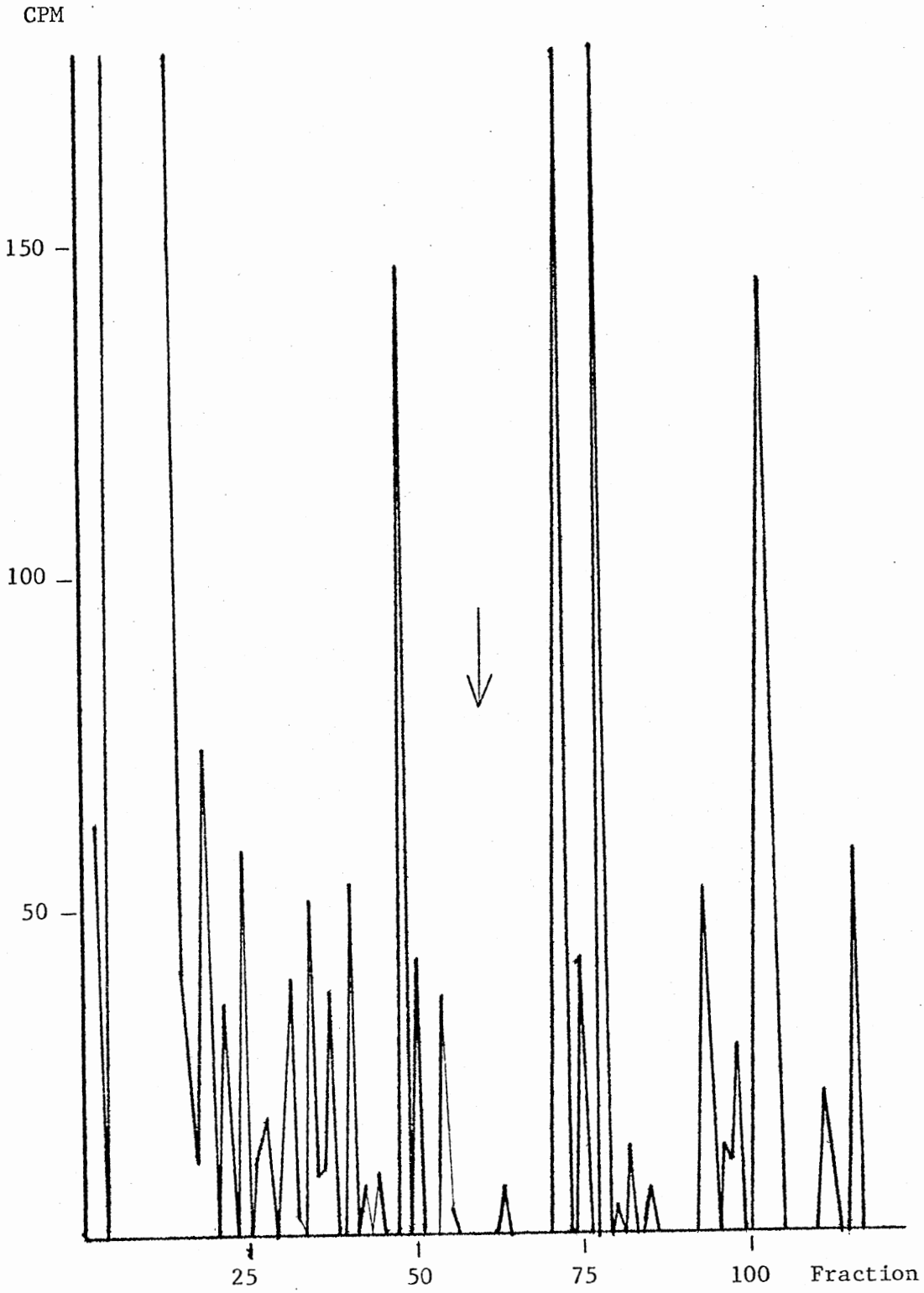
TABLE V

INHIBITION OF G6P UPTAKE BY NDGP*

[NDGP] (μM)	% Control
0.0	100
15.6	95
39.0	54
156.0	36
234.0	30

*[G6P] = 40 μM.

Figure 5. Column Affinity Chromatography. Arrow indicates switch from buffer T to buffer T + 1.0 mM G6P.


CPM
150
100
50
25
50
75
100
Fraction

RE215, and RE216 possessed two polypeptides which eluted with mannose-6-phosphate that the uhp^+ (uninduced) strain did not possess. These polypeptides have apparent molecular weights of 34,000 and 27,500 (Figure 6).

Batch chromatographic studies show that RE217 possessed a protein of molecular weight ∿21,000 which would elute only with glucose-6-phosphate in elution buffer. This protein would elute with a triton X-100 wash in RE213 studies (Figure 7). However, RE92 and RE215 also possessed this protein, but RE144 did not (Figure 8).

Figure 6. SDS PAGE of M6P Eluate of Affinity Chromatography. Lanes are left to right: RE92 (uninduced), RE144, RE215, and RE216.

Figure 7. SDS PAGE of Batch Affinity Chromatography. Lanes are left to right: RE213 whole cell, RE213 Buffer T wash, RE213 Buffer T + 1% Triton, RE213 Buffer T + 1% Triton + 1.0 mM G6P; Standards, last four lanes are washes of RE217 as with RE213.

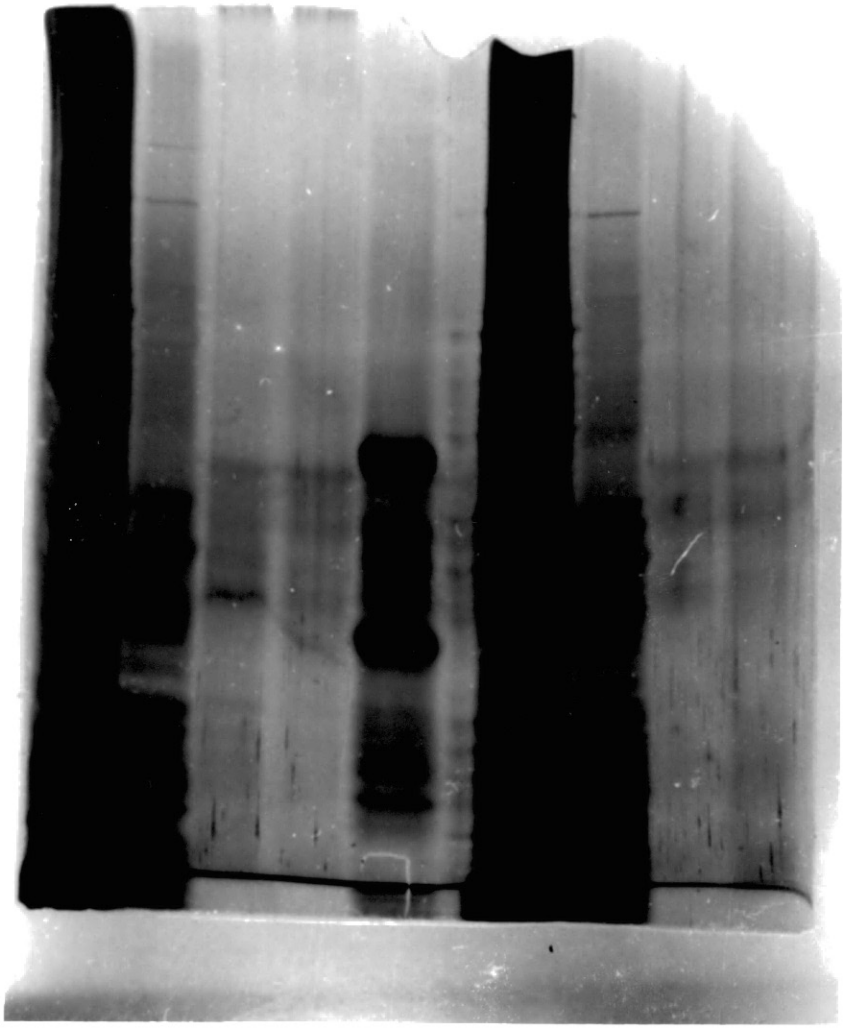


Figure 8. SDS PAGE of Affinity Chromatography. Lanes are left to right: MW standards, Tris·NaCl washes of RE217, RE215, RE144, and RE92, and G6P washes of RE217, RE215, RE144, and RE92.

CHAPTER IV

DISCUSSION

SDS-PAGE of inner membranes showed three proteins which could be associated with the uhp operon. RE92 (induced) and RE144 possessed proteins of 27,000, 35,000, and 64,000 molecular weight which RE92 (uninduced) did not possess. RE213 and daughter strains RE216 and RE217 with plasmids contained a protein of 21,000 molecular weight not present in RE144.

RE213 did not contain two proteins found in its daughter strains of 39,000 and 70,000 molecular weights. There was no evident difference between RE213 and daughter strains at 27,000 molecular weight range. The molecular weights of 35,000 and 64,000 found in uhp^+ (induced) and RE144 are within experimental error of those found in the plasmid strains.

The most likely candidate for the inducing protein is the 21,000 molecular weight protein. It is present in strains showing inducibility, RE92, RE216 and RE217. It is not present in the strains which show no increase in uptake of G6P under inducing conditions (RE144). The fact that it is present in the uhp^- strain can be accounted for if one considers that the mutation in RE213 is a point missense mutation in either the uhpT or uhpR gene. The protein would still be synthesized under both conditions but would be nonfunctional if the mutation is in the uhpR gene and causes an amino acid change affecting

the active site. This could be elucidated by further studies in the following manner. Since the mutation is a point mutation, studies on revertant strains capable of taking up G6P to determine if they are constitutive or wild type could clarify the location of the mutation. If the revertant strains are constitutive, then the mutation is in the uhpR gene. If it is in the uhpT gene, then reversion to wild type (inducible) system is expected.

R. Essenberg has mapped the plasmids in RE215, RE216 and RE217. pLC17-47 encompasses much of pLC40-33 and allot pLC35-4 but pLC40-33 and pLC35-4 do not overlap. Possible explanation of the uptake data and this information as well as the presence of or absence of the 21,000 molecular weight protein is as follows. The mutation in RE213 is in the uhpR gene. pLC17-47 (plasmid in RE217) contains functional uhpR and uhpT genes while RE216 plasmid (pLC35-4) contains only the uhpR gene. RE215 plasmid (pLC40-33) would contain only the uhpT gene. If this is true then a functional inducing protein would be present in RE216 and RE217 but not in RE215.

Shattuck-Eidens and Kadner (16) recently confirmed genetically the existence of two genes associated with uptake of hexose phosphates. One, designated uhpT, is responsible for the transport of hexose phosphates while the other, designated uhpR, is responsible for the induction protein. The separation of the translation products of these genes was attempted in this study via affinity chromatography. Separation was attempted on the basis that glucose-6-phosphate is the only substrate capable of inducing the system. Elution of the gel was performed with either F6P or M6P in buffer followed by elution with G6P in buffer. Since F6P and M6P are substrates for transport but not for induction,

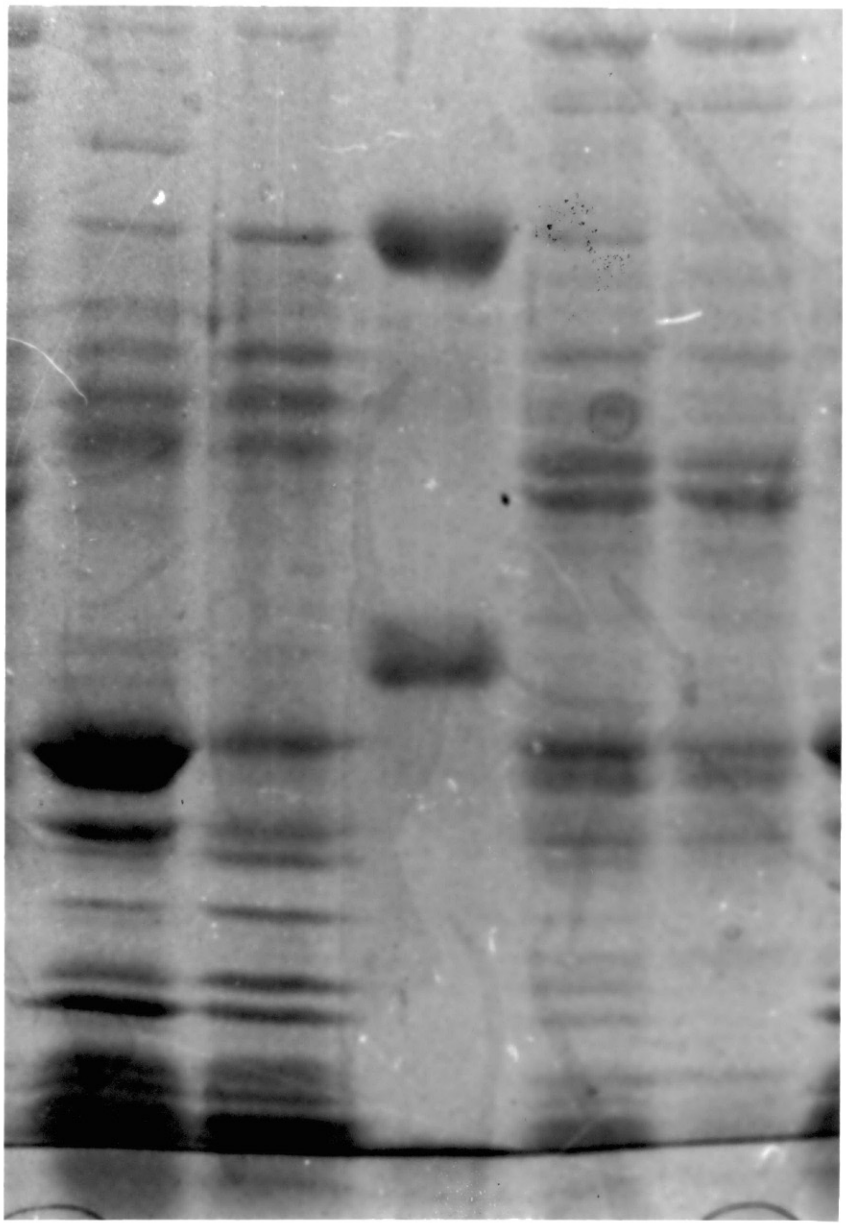
the transport protein(s) would be eluted with these substrates but the inducing protein would not. RE144, RE215 and RE216 M6P elutions contained two proteins of molecular weights 27,000 and 33,000. Elution of these strains with G6P showed a protein of molecular weight 21,000 as did elution of RE92 (uninduced).

This suggests that the transport protein is either the 27 Kd or 35 Kd protein and that the inducing protein is the 21,000 molecular weight protein. The fact that two proteins are eluted with M6P could be explained assuming that one protein is another membrane bound enzyme capable of using mannose-6-phosphate as a substrate. Other possible explanations are the requirement of two proteins aggregating together for transport or that the 35,000 protein is the precursor of the 27,000 molecular weight protein.

The requirement for two proteins in the transport process is favored here because of the existence of the 64-70,000 molecular weight protein in strains capable of transporting hexose phosphates. Boos (40) recently reported the inability of Laemmli buffer to separate aggregations of transport proteins involved in the transport of glycerol-3-phosphate. He found that heat treatment at 50^{o} could separate these aggregates but that heat treatment at 100^{o} would aggregate rather than separate these aggregates. Further study of this phenomenon would be beneficial to the elucidation of the nature of this 64,000 molecular weight protein.

Evidence for the involvement of a periplasmic component comes from osmotically shocked cells and spheroplast studies. Tentative evidence of a protein comes from SDS-PAGE of the 100 K x g supernatant (Figure 9) and affinity chromatography of whole cell extracts. The

Figure 9. SDS PAGE of RE144 Schnaitman Fractions. Lanes are from left to right: outer membranes, inner membranes, standards, sonication supernatant, and 100 K x g supernatant.



100 K x g supernatant in the Schnaitman procedure contains proteins of cell not present in membranes. There is a protein of 25,000 molecular weight in this fraction which absorbs so tightly to the affinity column that it can not be removed by 0.1 N HCl or 10 mM G6P. This protein may be a cytoplasmic protein involved in G6P metabolism so the evidence here is only sketchy at best. Further studies of this protein between uhp^c and uhp^- strains with binding studies and shock treatment of cells would help to establish the involvement of this protein with the uhp system. It may be that the functionality of the uhp mutants available in this lab is irrelevent to this protein as was shown to be the case with the arginine and ornithine transport systems (41). Celis isolated mutants defective in arginine and ornithine transport which possessed a functionally active binding protein.

A Possible Model

Models of systems should be able to explain the current known characteristics of the systems they represent. A model of the uhp system should incorporate the following characteristics. There appear to be three and maybe four proteins associated with the uhp system. One protein, 21,000 molecular weight, is involved in the induction process, two proteins are involved in the transport of hexose phosphates across the membrane and there may be a 25,000 molecular weight protein which has a binding function. The induction protein must be capable of interacting with the uhp gene either directly or indirectly allowing expression of the transport gene. External induction of the system is crucial in that it could explain

direct interaction of 21,000 molecular weight protein and no time lag between induction and transport and thus its location in the membrane.

The characteristics of the energy requirements show that the system receives energy from either electron transport or ATP indicating some form of coupling. Essenberg and Kornberg (1) have shown that the system follows the H^+-symport mold. H^+ equivalents are transported across membrane simultaneously with hexose phosphates.

Using these characteristics of the transport process, a model is presented (Figure 10). In step 1, the initial state of the system is seen. The inducing protein (21 Kd) is seen as being transverse in the membrane and spanning from one side to the other side of the membrane. The DNA is bound to the inducing protein at the inner surface of the cytoplasmic membrane.

In step 2, it is seen that the binding of external G6P (V) to the inducing protein causes two things to happen. The first result is the conformational change in the inducing protein. This in turn then results in the release of the DNA by the inducing protein. Once the DNA is free, transcription and translation of the uhpT gene can occur.

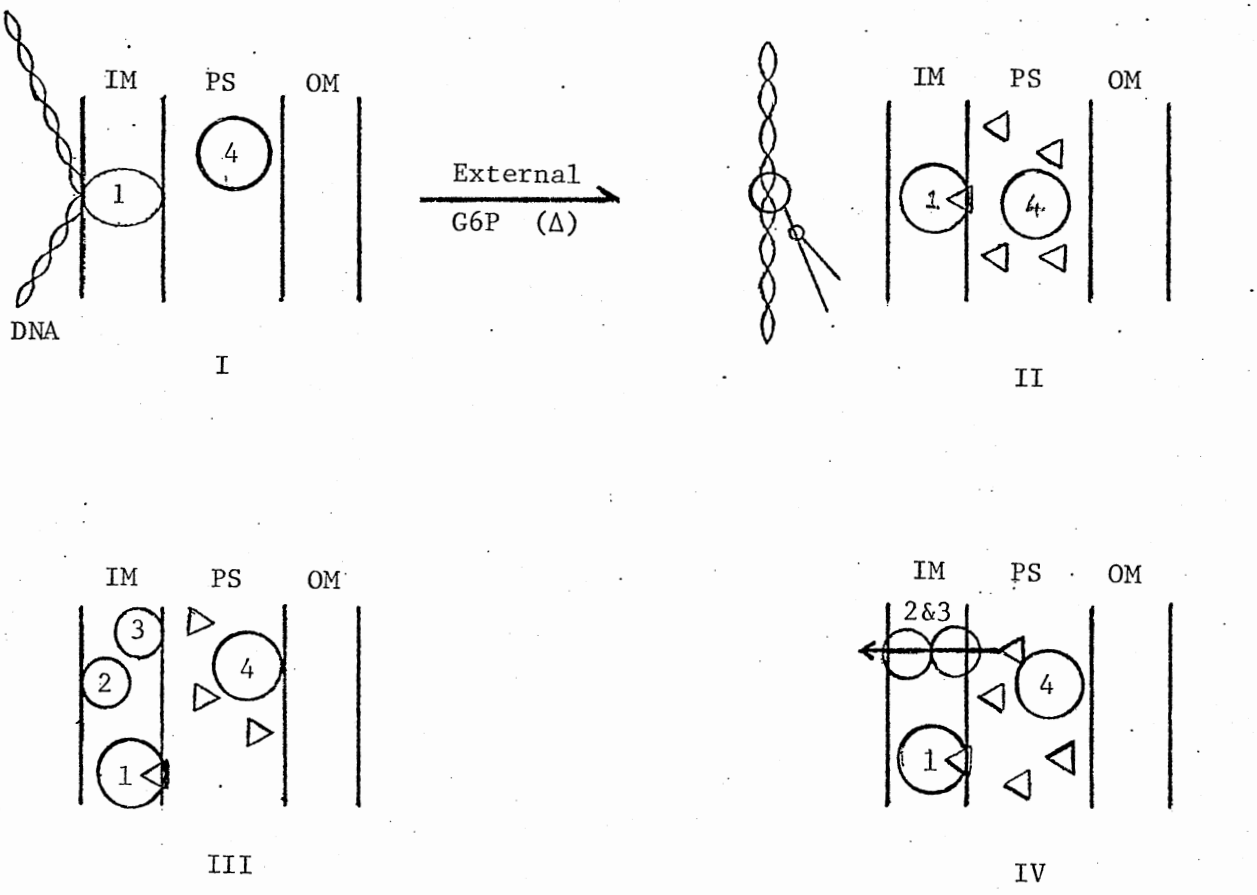
In step 3, the translation products of uhpT gene are incorporated in the cytoplasmic membrane. Step 4 shows aggregation of the transport proteins and transport of glucose-6-phosphate.

This model shows the 25,000 molecular weight protein but does not attempt to explain or show its involvement because there is no hard data linking it to the uhp system.

Future Studies

Obviously further studies will center around the isolation of

Figure 10. Model of Induction Process. IM = inner membrane; PS = periplasmic space; OM = outer membrane. Proteins: ① =21,000 Kd; ② =27,000 Kd; ③ =35,000 Kd; ④ =25,000 Kd.


IM
PS
OM
4
1
DNA
I
External
G6P (Δ)
IM
PS
OM
1
4
II
IM
PS
OM
3
2
1
4
III
IM
PS
OM
2&3
4
1
IV

components. This can most likely be accomplished with affinity chromatography. Once the components are isolated, several avenues could be taken to analyze functions of the individual components using the model supplied.

Binding of the 21,000 molecular weight protein to the uhp DNA could enhance the validity of the model. Performing studies similar to those by Wilcox et al. (23) with the araC gene product would be of significant use. Conformational change studies would also be useful.

Studies on the aggregation of the 27,000 and 35,000 proteins and analysis of the 64,000 molecular weight proteins could confirm the need for both in transport. Amino acid analysis of these components and time course studies of newly synthesized components after induction could show that transport proteins are synthesized after induction.

The involvement of a periplasmic space protein could further be established with osmotically shocked cells. This has not proved fruitful to date, but with the advent of silver staining of SDS-PAGE (37, 42), this could now be useful.

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VITA[1]

John Paul Wilder

Candidate for the Degree of

Master of Science

Thesis: IDENTIFICATION OF PROTEINS ASSOCIATED WITH UHP SYSTEM OF *E. COLI*

Major Field: Biochemistry

Biographical:

Personal Data: Born in DeRidder, Louisiana, November 6, 1949, the second son of Mr. and Mrs. E. C. Wilder.

Educational: Graduated from Delta Heritage Academy, Boothville, Louisiana as Salutatorian in June, 1967; attended McNeese State University in the fall, 1967; attended McNeese State University, fall, 1974 to graduation with Bachelor of Science degree in chemistry in December, 1977; entered Oklahoma State University in the fall, 1978; completed requirements for Master of Science degree at Oklahoma State University in December, 1982.

Professional: Outstanding Analytical Chemist at McNeese State University, 1977; Research Assistant, Oklahoma State University from fall, 1978 to December, 1982; Teaching Assistant at Oklahoma State University, spring, 1981.

Professional Organizations: Phi Lambda Upsilon.