

To the Graduate Council:

I am submitting herewith a thesis written by Rosemary B. Sanozky entitled "The Release of Periplasmic Proteins from Pseudomonas aeruginosa and Pseudomonas putida." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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THE RELEASE OF PERIPLASMIC PROTEINS FROM
PSEUDOMONAS AERUGINOSA AND
PSEUDOMONAS PUTIDA

A Thesis
Presented for the
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Rosemary B. Sanosky

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Finally, I am most grateful for good friends, inspiring teachers and a loving family. Immer wenn du denkst es geht nicht mehr, kommt von irgendwo ein Lichtlein her.

ABSTRACT

Attempts were made to isolate periplasmic glucose binding protein from Pseudomonas aeruginosa and Pseudomonas putida. Two basic shock procedures, derived from published methods, were employed. These were shocking with high magnesium or a modification of a sucrose-EDTA osmotic shock method. The activity of asparaginase, a known periplasmic enzyme in Pseudomonas, was used as a marker to evaluate the efficiencies of the different shock procedures. Release of glucose-6-phosphate dehydrogenase, a cytoplasmic marker, was used as a measure of viability and integrity of the cells that were shocked. Glucose binding activity was not detected in the shock fluid by equilibrium dialysis or by sephadex chromatography. Glucose uptake was compared in shocked vs. non-shocked cells of Pseudomonas aeruginosa PA 46 and RM 1. No significant decrease was found in the ability of the shocked cells to take up glucose. The inner and outer membranes of P. aeruginosa RM 1 were separated in order to attempt localization of glucose dehydrogenase, an exocyttoplasmic enzyme, and to test membrane fractions for binding activity. Studies with Pseudomonas putida involved using modifications of the sucrose-distilled water osmotic shock method. The degree of extractability of some known periplasmic enzymes was investigated. In P. putida, asparaginase was found to be tightly bound; it was released

to the same degree as glucose-6-phosphate dehydrogenase, the cytoplasmic marker. Attempts to preferentially release asparaginase by using high Tris concentrations and by preparing osmotically sensitive cells were not successful. Alkaline phosphatase, another periplasmic enzyme, was not released from P. putida cells grown in a phosphate-deficient medium, although some asparaginase activity was found in the shock fluid. These data support the hypothesis that in P. putida, and perhaps other Pseudomonas species, some "periplasmic proteins" are tightly bound and are only partially shockable, being released upon disruption of the cell.

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CHAPTER I

INTRODUCTION

The Periplasmic Space of Gram Negative Bacteria

The cell envelope of gram negative bacteria is a triple-layer structure composed of a cytoplasmic membrane, the peptidoglycan, and the outer membrane. The cytoplasmic membrane is a true bilayer with hydrophobic inner and outer faces. The outer membrane is also a bilayer, but the presence of lipopolysaccharide in the outer leaflet confers hydrophilic properties. It has recently been found (8) that certain proteins of the outer membrane aggregate to form aqueous pores through which hydrophilic molecules of less than 600 daltons can pass by diffusion. The peptidoglycan is found between the two layers; it is linked to the cell membrane by so-called adhesion points (6) and to the outer membrane by lipoprotein molecules. The periplasm comprises the total space between the two membranes, including the murein layer. The constituents of the periplasmic space are the topic of interest in this study.

The periplasmic space contains degradative enzymes which need to be sequestered from the cytoplasm; they degrade a wide variety of molecules that enter through the outer membrane. These enzymes include beta-lactamases, acetylases, phosphorylases and adenylases (19). In addition

to the hydrolytic enzymes, there are proteins, termed binding proteins, that bind nutrients for transport.

Periplasmic Binding Proteins

Periplasmic binding proteins of the enteric bacteria, Escherichia coli and Salmonella typhimurium have been fairly well characterized (21, 22). These releasable or "shockable" proteins are involved in the active, high affinity transport of various sugars, amino acids and inorganic ions. Sulfate binding protein and a histidine binding protein have been detected in Salmonella, while the following have been identified in E. coli: a phosphate binding protein, binding proteins for the amino acids, leucine, arginine, glutamine, cystine, and for the sugars galactose, arabinose, and maltose (21, 22).

Presumably, these binding proteins help to concentrate the solutes in the periplasmic region before they traverse the cell membrane. They are present in relatively large amounts (10^{-6} to 10^{-5} M) (22) and are apparently not involved in translocation processes, although they may contain a second binding site that could interact with a membrane component. The usual method for releasing periplasmic proteins is the Neu and Heppel osmotic shock procedure, first applied to E. coli (20). Using this method, release is accomplished by suspending cells in a sucrose-EDTA-Tris buffer solution followed by rapid resuspension in cold distilled water. This method has been used

successfully with E. coli and Salmonella; however, due to the EDTA sensitivity of Pseudomonas species, other methods have been applied.

Cheng, Ingram and Costerton (5) found that extraction of P. aeruginosa periplasmic proteins required treatment of cells with a high magnesium concentration (0.2 M), followed by suspension of the cells in 0.1 M Tris buffer pH 8.4. Using this ionic extraction procedure, Ingram and various coworkers (1, 5, 7) have localized some periplasmic enzymes in P. aeruginosa, namely, alkaline phosphatase, asparaginase and a ribonuclease.

The production of alkaline phosphatase is induced in growing cells in a phosphate-deficient medium. The enzyme cleaves the phosphate group from organic phosphate containing compounds, releasing inorganic phosphate. Asparaginase is a non-inducible enzyme which catalyzes the hydrolysis of L-asparagine at the amide group to yield ammonia.

The 0.2 M $MgCl_2$ extraction has not been totally successful. Not all periplasmic proteins were released to the same degree. Ingram et al. (1) had to vary the shock procedure, either utilizing high magnesium or 20% sucrose, to release a given enzyme. He concluded that in Pseudomonas, some periplasmic proteins are easily released, but others appear to be more tightly bound to an envelope component.

Binding Proteins in Pseudomonas aeruginosa

Recently, Stinson, Cohen and Merrick (25) investigated glucose and C₄-dicarboxylic acid binding proteins in P. aeruginosa. These proteins were found to be inducible and were extracted by treatment with 0.2 M MgCl₂, followed by a Tris buffer shock. Dicarboxylic acid binding protein synthesis is induced by growth on aspartate, alpha-ketoglutarate, succinate, fumarate, malate and malonate. It specifically binds succinate, fumarate and malate. The authors reported maximal production of glucose binding protein when cells were grown on glucose or, pyruvate and galactose, pyruvate and 2-deoxyglucose, and pyruvate and alpha-methylglucoside. Cells induced for glucose binding protein showed maximal uptake of ¹⁴C-labelled alpha-methylglucoside.

In a later paper, Stinson et al. (26) purified and characterized the glucose binding protein. It was found to be a glycoprotein with a molecular weight of approximately 44,500. Binding specificity studies showed that the purified glucose binding protein was specific for glucose.

The proteolytic properties of P. aeruginosa and the importance of amino acids in its metabolism prompted our investigation into the possible existence of amino acid binding proteins. But, none of the attempted shock methods described in this thesis (i.e., MgCl₂ extraction and the Neu and Heppel sucrose osmotic shock) provided evidence for

any binding activity. Therefore, much of the effort was devoted to developing an improved procedure for removing periplasmic proteins from Pseudomonas without damaging the cell.

Glucose dehydrogenase is produced by P. aeruginosa when grown in a glucose rich medium. Under these conditions (i.e., high glucose concentrations), the cells transport and primarily utilize gluconate produced by the oxidation of glucose (28). Glucose dehydrogenase has been assumed to be membrane associated (28). However, since the oxidation takes place in the exocyttoplasmic space, it could be associated with the outer membrane as well as the inner membrane.

An extension of the investigation with P. aeruginosa involved isolation of the inner and outer membranes, both to test for binding proteins in these fractions and to localize glucose dehydrogenase. The latter might supplant binding proteins as an alternative first step for entry into the cell.

Pseudomonas putida

When extraction with 0.2 M MgCl₂ proved unsuccessful with our strains of P. aeruginosa, an attempt was made to develop a workable shock procedure for Pseudomonas.

Pseudomonas putida was used because it was supposedly (23) more EDTA-resistant and the classical Neu and Heppel procedure could be employed (20).

The purpose of the work with P. putida was to test efficiencies of the modified Neu and Heppel procedures. This was accomplished by checking viability of shocked vs. unshocked cells and by testing the shock fluid for (a) levels of protein, (b) total asparaginase or alkaline phosphatase activity, known periplasmic enzymes in P. aeruginosa, and (c) levels of the cytoplasmic marker, glucose-6-P dehydrogenase. In addition, preparation of P. putida spheroplasts was attempted in order to clarify results obtained in the shock experiments.

CHAPTER II

MATERIALS AND METHODS

Organisms

Two species and various strains of Pseudomonas were used in this study. These were: P. aeruginosa RM 1, a PAO strain; P. aeruginosa PA 46, a methionine auxotroph of RM 1 (both of these strains were provided by Dr. R. V. Miller, The University of Tennessee, Knoxville); P. aeruginosa PAO 1 ATCC 15692, provided by Dr. M. W. Stinson, School of Medicine, State University of New York at Buffalo; P. aeruginosa UT 5611, a protease-free strain which was a gift of Dr. J. M. Woodward, The University of Tennessee, Knoxville; P. aeruginosa PA 34, a lipopolysaccharide mutant which was kindly provided by Dr. D. F. Day, Microbiology Department, University of Guelph, Guelph, Canada; and P. putida ATCC 17421, which was purchased from the American Type Culture Collection.

Media and Growth of Cells

All of the P. aeruginosa strains were routinely grown at 35° C. Pseudomonas putida was grown at its optimum temperature of 26° C. Stock cultures were kept in Luria Broth, composed of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride, pH 7.0. Unless otherwise specified, cultures for all experiments were grown

in Cascieri and Mallette minimal medium (3) pH 7.0 composed of the following inorganic salts: $(\text{NH}_4)_2\text{SO}_4$, $7.56 \times 10^{-3}\text{M}$; K_2HPO_4 , 0.06M; KH_2PO_4 , 0.033 M; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $4 \times 10^{-4}\text{M}$. A separately autoclaved solution of either glucose or sodium citrate was added to a concentration of 0.02 M.

Cells were grown overnight (16-18 hours) on a New Brunswick rotary waterbath shaker. Ten milliliters of cells were transferred into 100 mls of new medium and harvested for the shock procedure when mid-logarithmic growth was reached (after 4-8 hours or 120 Klett Units on a Klett-Summerson Photoelectric Colorimeter with a red, #62 filter).

Phosphate-deficient medium. For induction of alkaline phosphatase, cells were grown in phosphate-deficient medium (5) of the following composition: 0.12 M Tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer pH 7.2; 0.02 M NH_4Cl ; 0.02 M KCl ; $1.6 \times 10^{-3}\text{M}$ $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5% proteose peptone, and 0.1% glucose.

Membrane separation medium. The method of Hancock and Nikaido (12) was adopted for membrane separation experiments. The medium used was 0.8% (w/v) tryptone, 0.5% yeast extract (w/v) and 0.5% (w/v) NaCl . Cells were grown overnight (16-17 hrs.) with shaking at 35°C and diluted 11-fold into a total of 2 liters fresh medium. Cells were harvested after an additional 6 hours when O.D.₅₉₅ reached 1.0 as measured with a Spectronic 20 Spectrophotometer.

Uptake Studies

A comparison of glucose uptake in glucose-grown, shocked versus unshocked cells was done with PA 46 and RM 1. To test for inducibility of transport, glucose uptake experiments were performed with citrate or glucose grown cells. Uptake studies involved incubating a standardized suspension of cells (135 Klett units measured with a red #62 filter) with 222 $\mu\text{g/ml}$ chloramphenicol in transport wash buffer (0.05 M potassium phosphate buffer, pH 7.0, 4.92 mM MgCl_2). After 15 minutes, labelled substrate (4.95 $\mu\text{Ci}/\mu\text{mole}$) was added to cells at a final concentration of 0.1 mM and samples taken at 30 sec intervals which were then applied to nitrocellulose filters. Filters were washed with transport wash buffer, then transferred to scintillation vials with 5 mls of ScintiPrep Toluene counting fluid and counted in a Beckman LS-100 counter. The counting fluid was prepared by diluting 20 mls of ScintiPrep with one quart of toluene. Uptake of substrate was expressed as nmoles per minute per mg protein.

Shock Procedures

PA 46 and RM 1 were shocked using the extraction method of Stinson et al. (25). This involved growing cells to early logarithmic phase (at O.D.₅₉₀ 0.23-0.30) then centrifuging for 10 minutes at 16,300 X g at room temperature. The pellet of cells was concentrated 100-fold with extraction buffer [0.2 M MgCl_2 - 0.05 M Tris (hydroxymethyl)

aminomethane-HCl buffer pH 8.5] and incubated for 30 minutes at 22° C with moderate shaking on a rotary shaker. After centrifugation at 4° C, the supernatant was saved and the pellet was resuspended in the same volume of distilled water and shaken for 30 minutes at 22° C. After centrifugation, this supernatant and the magnesium extract were assayed for protein and binding activity.

P. putida and PA 46 were shocked according to the Neu and Heppel procedure (20) for E. coli. This entailed suspending a pellet of mid-log phase cells (1 g wet wt.) in 80 mls 20% sucrose-0.03 M Tris pH 8.0 with 0.001 M EDTA for 10 minutes with stirring. This was followed by a 10 minute centrifugation at 16,300 X g, 4° C and rapid re-suspension of the pellet in 80 mls of ice-cold distilled water with shaking for 10 minutes. The supernatant of the final centrifugation was termed the cold water shock fluid and should contain periplasmic proteins. The procedure was later carried out with some modifications, including omission of EDTA. Shock fluids were concentrated 12-15-fold by Amicon ultrafiltration cones or by Amicon Filtration Stirred Cell with a molecular weight cut-off of 10,000 (PM 10).

Membrane Preparation-Separation of Inner and Outer Membranes

The method of Hancock and Nikaido (12) was used to separate the inner and outer membranes of RM 1. All work was done at 4° C. Two liters of cells (250 mls yields

approximately 1.6 g (wet wt.) were washed twice with 30 mM Tris buffer pH 8.0 with 0.5 mg DNase and 0.5 mg RNase. The cells were then disrupted by two passages through a French Pressure Cell at 15,000 psi followed by addition of 1 ml of lysozyme (1 mg/ml). Ten minutes later the protease inhibitor, phenylmethyl sulfonyl fluoride (solubilized in iso-propanol) was added to a final concentration of 1 mM. Cell debris was removed by centrifugation at 1,085 X g for 10 minutes. The supernatant was diluted by addition of 7 mls of the Tris buffer, and 4-5 mls of the suspension was layered onto each of five sucrose step gradients containing 1 ml 70% (wt/vol) sucrose and 6 mls 15% (wt/vol) sucrose in Tris buffer. These tubes were centrifuged at 183,000 X g in a Beckman SW 41 rotor for one hour. The bottom 2 mls were collected and applied to new step gradients composed of 1 ml 70% (wt/vol) sucrose, 3 mls 64% (wt/vol) sucrose, 3 mls 58% (wt/vol) sucrose, and 3 mls 52% (wt/vol) sucrose in Tris buffer and centrifuged overnight (14-1/2 hours) at 183,000 X g. Four bands were observed after this centrifugation. These bands were collected by suction from top to bottom: The inner membrane (IM) (deep red); the "M" band (pale orange); the outer membrane (OM₂) (paler orange); and the outer membrane (OM₁) (translucent white). According to Hancock and Nikaido (12), the OM₁ and IM were fairly pure preparations, the OM₂ fraction had some inner membrane enzyme contamination and the M fraction had high quantities

of both inner and outer membrane markers. Each fraction was diluted with distilled water to a final volume of 12 mls and centrifuged at $177,700 \times g$ in a Sorvall 650 rotor for one hour. The pellets were resuspended in enough water for a final volume of 8 mls and dialyzed against a liter of 0.01 M sodium phosphate buffer pH 8.3 for 18 hours.

Preparation of Spheroplasts

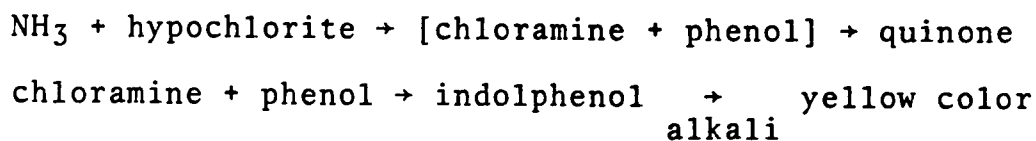
Modifications of the method of Hoshino and Kageyama (14) were employed. Mid-log phase cells of P. putida were harvested by centrifugation at $10,000 \times g$ for 10 minutes at $25^{\circ} C$ and suspended to a concentration of 3×10^{10} cells/ml (135 Klett units, red #66 filter) in 10 mM potassium phosphate buffer pH 7.0 containing 0.75 M sucrose, 10 mM $MgSO_4$ and 0.5 mg/ml lysozyme. A major modification was the addition of EDTA to 1 mM. The suspension was then rapidly chilled in ice for 10 minutes and gradually warmed with slow shaking to $30^{\circ} C$ with a $38^{\circ} C$ waterbath. This was followed by a 30 minute incubation at $30^{\circ} C$ with gentle shaking. Samples of the suspension were checked for the presence of rounded spheroplasts and an increase in cellular debris was observed after addition of distilled water. The spheroplasts were collected by centrifugation at $10,000 \times g$ for 30 minutes at $4^{\circ} C$. The supernatant was called the "spheroplast" medium. The pellet was resuspended in 0.5 ml of 10 mM potassium phosphate buffer pH 7.0 with 0.75 M sucrose. Cells were lysed by a rapid 50-fold dilution in

10 mM potassium phosphate buffer pH 6.8 containing 1 mM MgSO_4 , 20 $\mu\text{g/ml}$ DNase and 20 $\mu\text{g/ml}$ RNase. After a 30 minute incubation at 25° C with gentle stirring, the suspension was centrifuged at 30,000 X g for 30 minutes at 4° C. The resulting supernatant was called the "spheroplast lysate." Enzyme assays were run on the two supernatants. In one set of experiments, the sucrose in the spheroplast medium supernatant was eliminated by dialysis against 0.01 M sodium phosphate buffer pH 8.3 with 0.001 M mercaptoethanol at 4° C for 19 hours. The dialysate was ultrafiltered in an Amicon Stirred Cell with a PM 10 filter.

Enzyme Assays

Shock experiments. Asparaginase activity was detected by using a modification of the Yellin-Wriston procedure (29) coupled to the Berthelot Ammonia reaction (4). The reaction was started by adding 0.25 ml of 0.04 M L-asparagine in 0.1 M sodium borate buffer pH 8.5 to 0.75 ml of enzyme solution. At 10, 20, and 30 minute time intervals, at 37° C, the reaction was stopped by addition of 0.25 ml 15% trichloroacetic acid (wt/vol). Liberated ammonia was detected by the Berthelot method (4). This involves adding 3.75 mls of Phenol color reagent followed by 3.75 mls of Alkali-hypochlorite reagent. The Phenol color reagent was prepared by mixing 60 mls of phenol and 0.25 g sodium

nitroprusside per liter. Preparation of the Alkali-hypochlorite reagent involved mixing 25 g sodium hydroxide and 40 mls of commercial bleach (Clorox) and diluting, with water, to one liter. The addition of these reagents yields the following reaction after 20 minutes at 37° C:



This color was measured in a Klett-Summerson Photometer using a green #54 filter. Ammonium chloride in 0.1 M sodium borate buffer (pH 8.5) was used as a standard. A unit of activity is defined as that amount of enzyme which will catalyze the formation of one micromole of ammonia per hour. Specific activity is expressed as units per mg protein.

The assay used for detection of glucose-6-P dehydrogenase activity was similar to the one described by Malamy and Horecker (18). This enzyme catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate with the concomitant reduction of NADP to NADPH. The activity is followed by observing the increase in absorbance of the reduced coenzyme at 340 nm on a Spectronic 20. The blank was 1.5 ml of water mixed with 1.5 ml of glucose-6-phosphate substrate. The substrate contained 0.1 M Tris buffer pH 7.7; 0.02 M magnesium chloride; 0.6 mM nicotinamide adenine dinucleotide phosphate (NADP); 4.0 mM glucose-6-phosphate and 0.02 M 2-mercaptoethanol. To monitor activity of cell fractions, 1.5 ml substrate was mixed with 1.5 ml of enzyme

and absorbance at 340 nm was followed with time. Units of enzyme are expressed as units per mg protein where one unit equals one O.D. unit change per hour.

Fractions from phosphate-deprived cells were tested for alkaline phosphatase activity by the Torriani method (27). The enzyme catalyzes the cleavage of p-nitrophenyl phosphate (NPP) to p-nitrophenol (NP) which absorbs at 420 nm. The substrate solution contained 6.6×10^{-3} moles of NPP dissolved in one liter of 0.6 M Tris HCl buffer at pH 8.2. The blank was 2.7 mls of substrate mixed with 0.3 ml water. Three tenths of a milliliter of enzyme was added to 2.7 ml substrate to start the reaction and the absorbance at 420 nm was followed with time. One unit of enzyme produces an O.D. change of 1.0 per hour.

Membrane preparations (assays performed on dialyzed membrane fractions). Succinate dehydrogenase was used as an inner membrane marker. A modification of the Kasahara and Anraku method (15) was used. This modification involved elimination of one assay component, phenazine methosulfate (PMS) and doubling the concentration of another, 2,6-dichlorophenol indophenol (DCPIP). The three ml reaction mixture contained 50 mM Tris buffer pH 8.0, 4 mM KCN, 30 mM sodium succinate, 0.08 mM DCPIP, and 0.2 mg membrane protein. The first three components were placed in a cuvette and incubated for 5 minutes at 25° C. Then the enzyme was added and the incubation continued for another minute. The reaction

was started by adding DCPIP and the decrease in absorbance at 600 nm was followed on a Beckman recorder attached to a Perkin-Elmer Coleman 124 Double Beam Spectrophotometer. A cuvette minus enzyme was used as a reference. One unit of succinate dehydrogenase activity was defined as the amount of enzyme catalyzing the reduction of one nanomole of DCPIP in one minute. The value for the extinction coefficient of DCPIP used was $2.2 \times 10^4 \text{ cm}^{-1} \text{ mole}^{-1}$, pH 8.0.

For glucose dehydrogenase activity a modification of the Hauge method (13) was used. This entailed doubling the concentration of the artificial electron acceptor DCPIP. The reaction mixture was prepared by combining into a 1 cm cuvette: 1.5 ml of 0.1 M sodium phosphate buffer pH 6.0, 0.2 ml DCPIP ($1.2 \mu\text{mole/ml}$ water), 0.2 mg enzyme protein and enough water for the final volume of 3 mls. After one minute incubation at room temperature (in order to exhaust the endogenous reducing power), the reaction was started by adding the substrate, 0.1 ml of 0.6 M glucose. The reduction of the dye was monitored by following the decrease in the absorbance at 600 nm at room temperature on a Perkin-Elmer Coleman 124 Double Beam Spectrophotometer with a Beckman recorder. A cuvette without enzyme was used as a reference. One unit of enzyme is defined as the amount of enzyme that oxidizes one μmole of glucose per minute. This corresponds to a change in absorbance at 600 nm per minute of 5.0.

Assay of Binding Activity

Binding activity of concentrated shock fluid or membrane fraction preparations was determined by equilibrium dialysis as described by Stinson et al. (25) for P. aeruginosa. Three tenths ml of shock fluid or membrane preparation was placed in a dialysis bag and dialyzed for 24 hours at 4° C against 30-35 mls of 0.05 M sodium phosphate buffer, pH 7.0 containing 0.02% sodium azide and radioactively labelled substrate added to the outside buffer (2 μ M or 5 μ Ci per 30 mls buffer). The substrates tested were: U- ^{14} C-glucose (10 mCi/mmole); U- ^{14} C-glucose (316 mCi/mmole); U- ^{14} C-gluconate (5.6 mCi/mmole); U- ^{14} C-glutamine (7.4 mCi/mmole); and U- ^{3} H-methionine (0.7 Ci/mmole). Binding was measured by taking 100 μ l samples from inside the dialysis bags and from the outside buffer and counting each with 5 mls of Bray's solution.

Binding activity was also tested by mixing labelled glucose and shock fluid from PA 46 and applying to a G-15 Sephadex column. Fractions were monitored for radioactivity and protein content.

Viability

Shocked and unshocked cells were suspended in 0.05 M potassium phosphate buffer pH 7.0 to an O.D. of 135 Klett units (0.2 mg/ml protein) using a red #66 filter on a Klett-Summerson photometer. Serial dilutions were performed in Cascieri and Malette Minimal Medium and samples were plated

onto nutrient agar plates. Plates were counted after 18-24 hours at either 35° C or 25° C. Percent viability was calculated by comparing plate counts of shocked cells with those of unshocked cells.

Disruption of Cells

Shocked and whole (unshocked) cells were disrupted by passing cells twice through a pre-cooled French Pressure Cell at 15,000 lb/in².

Protein Determination

The protein content of cell fractions was determined either by the Bio-Rad (2) or Lowry method (16).

CHAPTER III

RESULTS

A. Pseudomonas aeruginosa

Glucose Uptake

Glucose uptake was compared in Mg++ shocked versus unshocked (normal) cells of both PA 46 and RM 1. No significant difference was found in their ability to take up glucose. Figure 1 shows the results with PA 46. Similar results were obtained with RM 1 (Figure 2).

The glucose uptake system was induced in glucose-grown cells but not in citrate-grown cells (Figure 3). Citrate cells showed no increase in counts compared to glucose-grown cells which took up 15.85 nanomoles glucose per minute per milligram protein. The term "uptake" is intended to include metabolism of the substrate. In order to study transport of glucose in Pseudomonas, one would have to use the analog, methyl-alpha-glucoside, which is not metabolized.

Shock Experiments Results

Several Pseudomonas strains were shocked according to the Stinson, Cohen and Merrick procedure (25): PA 46, RM 1, PAO 1 ATCC 15692 and UT 5611. Strain 15692 was used in an attempt to reproduce Stinson's results. The protease-free strain, UT 5611, was shocked to eliminate the

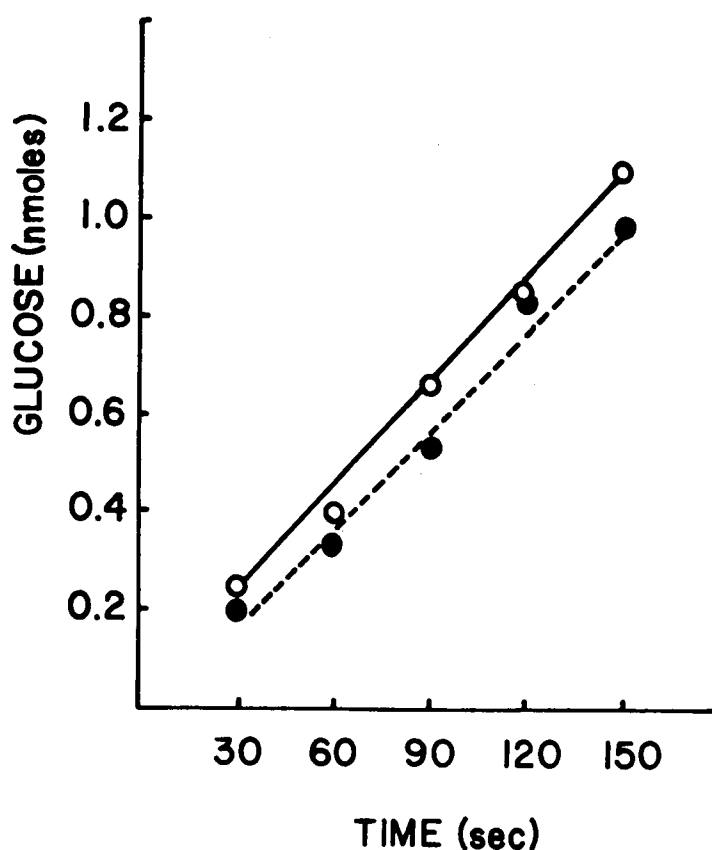


Figure 1. Glucose uptake in *Pseudomonas aeruginosa* PA 46. Cells were grown in minimal medium and glucose. Half of the culture was shocked according to the magnesium extraction method of Stinson et al. (25) and the other half remained refrigerated. Both cultures were suspended to 135 Klett units with Transport wash buffer and uptake initiated with addition of 4.95 $\mu\text{Ci}/\mu\text{mole}$ glucose. Each sample contained 0.02 mg protein.

●---● unshocked cells-rate of uptake-16.55 $\mu\text{moles}/\text{min}/\text{mg}$.

o—o shocked cells-rate of uptake-21.10 $\mu\text{moles}/\text{min}/\text{mg}$.

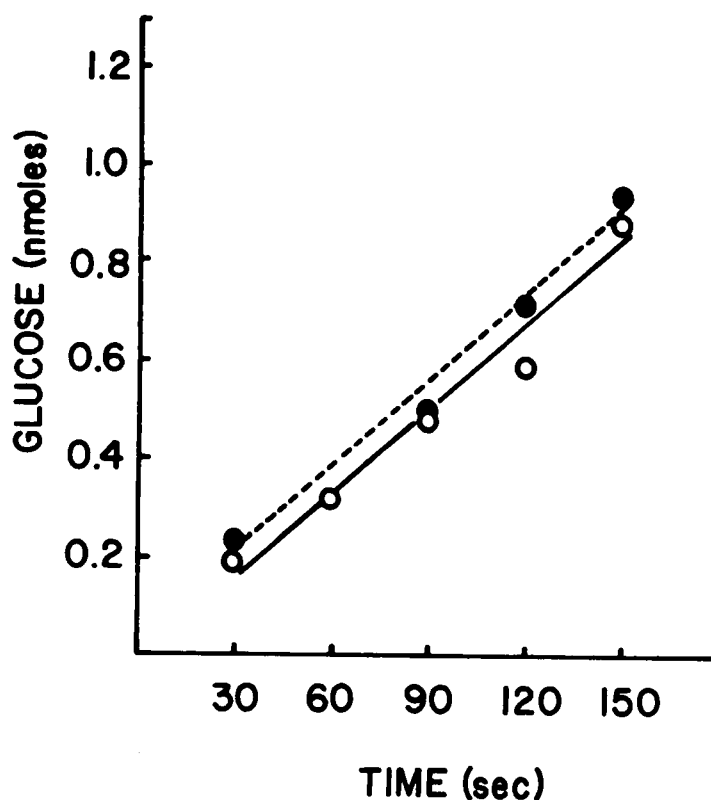


Figure 2. Glucose uptake in Pseudomonas aeruginosa RM 1. Cells were grown in minimal medium and glucose. The culture was split into two fractions. Half were shocked according to the extraction method of Stinson et al. and the other half was refrigerated during the extraction. Both cultures were suspended to 135 Klett units with Transport wash buffer and uptake initiated with the addition of 4.95 $\mu\text{Ci}/\mu\text{mole}$ glucose. Each sample contained 0.02 mg protein.

●---● unshocked cells-rate of uptake-13.95 $\mu\text{moles}/\text{min}/\text{mg}$.

○—○ shocked cells-rate of uptake-14.55 $\mu\text{moles}/\text{min}/\text{mg}$.

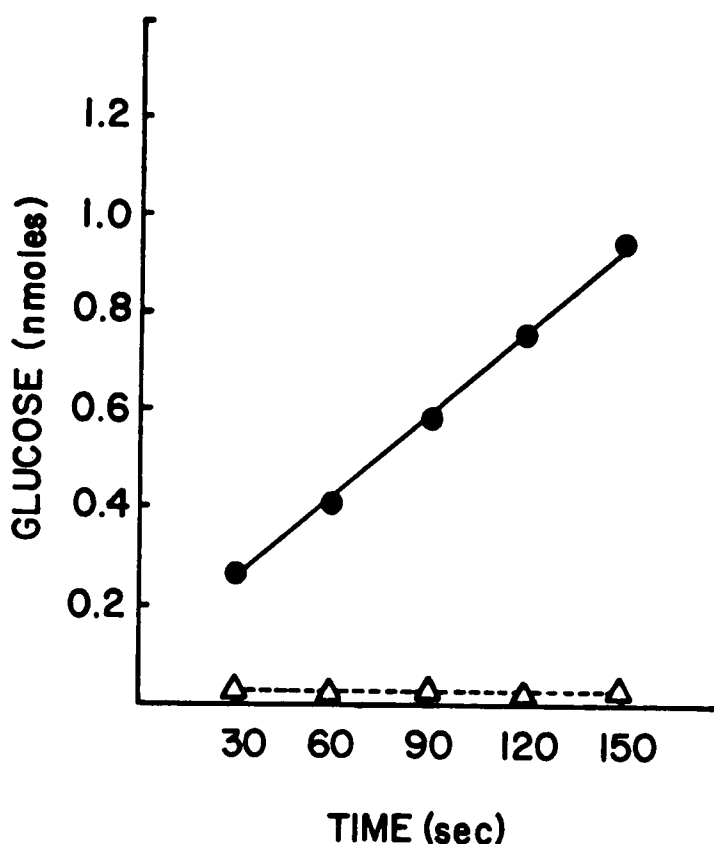


Figure 3. Glucose uptake in *Pseudomonas aeruginosa* PA 46-citrate vs. glucose-grown cells. Cells were grown in minimal medium with either glucose or citrate as the carbon source (20mM). These unshocked cells were then adjusted to the standard suspension of 135 Klett units. Uptake was initiated with the addition of labelled glucose (4.95 $\mu\text{Ci}/\mu\text{mole}$). Each sample contained 0.02 mg protein. The glucose-grown cells took up 15.85 $\mu\text{moles glucose}/\text{min}/\text{mg}$.

●—● glucose-grown cells.

Δ---Δ citrate-grown cells.

possibility that a protease was destroying the binding proteins.

Little or no protein was found in the shock fluids and no glucose or amino acid binding activity was detected, either by equilibrium dialysis or by sephadex chromatography. A search for a repeatable shock method led to utilization of a modified Neu and Heppel procedure for the EDTA-sensitive P. aeruginosa. The goal was to optimize extraction of periplasmic proteins without affecting viability. Indicators used to judge the degree of success of the shock procedure were measurement of the following in the concentrated shock fluid: (a) high protein content, (b) high asparaginase activity, (c) low glucose-6-P dehydrogenase activity.

In this study the lipopolysaccharide mutant PA 34 was used. The modified shock procedure involved suspension of one gram cells (wet wt.) in 80 mls of 20% sucrose-0.03 M Tris or potassium phosphate buffer, pH 8.0. After a 10 minute, 24° C incubation and centrifugation, the pellet was resuspended in 80 mls of cold distilled water with various magnesium concentrations. The results are presented in Table 1. P. aeruginosa 34 appears to be sensitive to Tris buffers. At 0.005 M Mg⁺⁺, an average of a 51% increase in viability was noted when phosphate was used in place of Tris. Although more protein is extracted with Tris buffer, the asparaginase activity is low. The actual effect of Tris is not known. Perhaps Tris loosens the anchoring lipoprotein

TABLE 1
RESULTS OF SHOCKING PA 34

Buffers and Temp. ^a	Mg ⁺⁺ Conc. (Molar)	Percent Viability	Protein Conc. in Original (mg/ml)	Asparaginase Activity Units/mg	Glucose-6-P Dehydrogenase Activity Units/mg
Tris	0.005	33	0.014	4.2	0.22
PO ₄ ⁼	0.005	76	0.006	5,330.0	2.44
PO ₄ ⁼	0.005	58	0.007	5,490.0	1.51
PO ₄ ⁼	0.01	60	0.003	1,930.0	0.33
PO ₄ ⁼	0.001	55	0.022	4,750.0	1.15

^aRoom temperature.

of the outer membrane and when cells are subjected to distilled water in the shock procedure, viability is decreased. For this organism, the optimum conditions for shocking the cells involves using 0.005 M Mg^{++} because viability is retained and asparaginase activity is fairly high, compared to other conditions.

Growth Temperature--Effect on Shocking PA 34

It was hypothesized that an alteration in the growth temperature might affect the extractability of periplasmic proteins of this LPS-defective mutant. Shock results of PA 34 grown at temperatures 5° C lower and higher than the optimum of 35° C are shown in Table 2. By shifting the growth temperature from 30° to 40° C, viability was lowered, but a 10-fold increase in the amount of extracted protein was noted. There was also a significant increase in the activities of asparaginase and glucose-6-phosphate dehydrogenase. If one compares these results with those of PA 34 grown at 35° C (Table 1), where 0.005 M Mg^{++} phosphate buffer was used, the following observations can be made: with growth at 30° C the viability is higher but less protein is shocked out and there is no enzyme activity; with cells grown at 40° C (compared with 35° C), viability is slightly lower, but more protein is released. The asparaginase activity is approximately the same in both cases but the glucose-6-P dehydrogenase activity is rather high. These data suggest that at 40° C, the outer wall of

TABLE 2
EFFECT OF GROWTH TEMPERATURE IN SHOCK OF PA 34

Growth Temp.	Buffer ^a	Mg ⁺⁺ Conc. (Molar)	Percent Viability	Protein Conc. in Original (mg/ml)	Asparaginase Activity Units/mg	Glucose-6-P Dehydrogenase Activity Units/mg
30° C	PO ₄	0.005	145	0.0013	0.0	0.00
40° C	PO ₄	0.005	70	0.013	5,045.0	3.43

^aTemperature of the wash buffer was 25° C.

Pseudomonas is weakened, which not only facilitates release of periplasmic proteins, but also somehow affects the cell membrane, giving the high glucose-6-P dehydrogenase activity.

Membrane Enzyme Assays--RM 1

A possible explanation for the lack of glucose binding protein in P. aeruginosa is the presence of glucose dehydrogenase. Activity of glucose dehydrogenase might obviate the necessity for a binding protein because the former enzyme would exocyttoplasmically oxidize glucose to gluconate for further metabolism. In order to localize the enzyme, and to determine whether or not binding proteins are membrane-associated, the inner and outer membrane fractions were tested for glucose dehydrogenase and binding activities.

To localize glucose dehydrogenase, enzyme assays were performed on separately dialyzed membrane fractions. Succinate dehydrogenase was used as a marker for cytoplasmic membrane enzymes. Two-tenths milligram membrane protein [as determined by the Lowry method (16)] was used in all assays. The results are outlined in Table 3. Binding activity of membrane fractions was tested by equilibrium dialysis against labelled substrate.

Both experiments show that the activity of succinate dehydrogenase is 100-fold greater in the inner membrane than

TABLE 3
 ENZYME ASSAYS ON MEMBRANE FRACTIONS OF RM 1

Exp	Fraction	Succinate Dehydrogenase Specific Activity moles/min/mg	Glucose Dehydrogenase Specific Activity Units/mg
1	Inner Membrane	3.07×10^{-2}	0.018
	Outer Membrane ₁	6.80×10^{-4}	0.016
2	Inner Membrane	6.82×10^{-3}	0.0073
	"M"	N.D.	0.0040
	Outer Membrane ₂	N.D.	0.0032
	Outer Membrane ₁	5.58×10^{-5}	0.0032

N.D. = Not determined.

the outer membrane, as expected. The activity of glucose dehydrogenase appears to be equally distributed in both the inner and outer membrane fractions. With respect to binding activity, no glucose or gluconate binding activity was detected in the membrane fractions.

B. Pseudomonas putida

Modifications of Shock Procedure

Since P. putida is claimed to be less sensitive to EDTA than P. aeruginosa (6, 23), the Neu and Heppel shock procedure (for E. coli) was applied to P. putida, as was reported by Reber et al. (23). However, survival of our strain of P. putida was low in 0.001M EDTA. The shock pellet was very slimy, indicating lysis and DNA release; most cells were lost upon filtration. Therefore, various magnesium ion concentrations ($5 \times 10^{-5}\text{M}$, $5 \times 10^{-4}\text{M}$, $5 \times 10^{-3}\text{M}$, and $1 \times 10^{-3}\text{M}$) were incorporated into the EDTA step in order to observe a possible protective effect. Protection was not demonstrated. The presence of EDTA consistently caused slimy pellets and clogged filters, so it was omitted from the shock procedure. This altered procedure, i.e., 20% sucrose-0.03M Tris and rapid suspension in distilled water, also lowered viability, so various magnesium concentrations were included in the distilled water step in order to increase viability. Phosphate buffer was substituted for Tris buffer in an attempt to further improve viability.

The results suggest first that, like P. aeruginosa, P. putida is also sensitive to Tris buffer and secondly, cellular integrity is better retained when phosphate buffers are used for washing and shocking cells. By looking at Table 4, one can see the improvement in viability when cells were exposed to phosphate buffer. For example, at 0.001M Mg^{++} , when compared to Tris treatment, a 50% increase in viability with phosphate buffer was noted; at 0.01M Mg^{++} , viability increased by approximately 35%. In general, as the magnesium concentration was raised, viability, amount of protein in the shock fluid and asparaginase activity increased.

Distribution of Asparaginase

In an attempt to calculate the percent of total asparaginase activity released during osmotic shock, shocked cells and non-shocked, whole cells were disrupted by a French Pressure Cell and the extracts (shocked cell extract and whole cell extract) were assayed for asparaginase and glucose-6-P dehydrogenase activity. The surprising results (Table 5) show that asparaginase is found not only in the periplasmic shock fluid but that almost all activity is associated with the cell-free extracts. Only 3-5% of total asparaginase was released from the cell. The data in Table 5 show that about 6% of total glucose-6-P dehydrogenase activity is released during the shock procedure. The fact that asparaginase is

TABLE 4
RESULTS OF SHOCKING P. PUTIDA^a

Exp	Buffer (Room Temp.)	Mg++ Conc. (Molar)	Percent Viability	Protein Conc. in Original Shock Fluid (mg/ml)	Asparaginase Activity (Units/mg)
1	Tris	0.001	39	0.045	2,133.0
2	Tris	0.001	29	0.061	1,180.0
3	Tris	0.01	57	0.018	360.0
4	PO ₄ ⁼	0.01	129	0.0176	N.D. ^b
		0.005	126	0.0296	N.D. ^b
		0.001	67	0.0299	N.D. ^b
5	PO ₄ ⁼	0.005	67	0.049	1,080.0
6	PO ₄ ⁼	0.01	86	0.015	615.0

^aThe standard shock system contained 0.03M buffer-20% sucrose; the subsequent distilled water step contained Mg++ as indicated above.

^bN.D. = Not determined.

TABLE 5

TOTAL CELL ASPARAGINASE AND GLUCOSE-6-P DEHYDROGENASE
IN P. PUTIDA -GROWN ON GLUCOSE

Exp	Fraction	Protein		Glucose-6-P Dehydrogenase				Asparaginase		
		Total Units (mg)	Percent of Total	Specific Act. Units/mg	Total Units	% of Total Act. ^a	Total Act. ^a	Specific Act. Units/mg	Total Units	% of Total Act. ^a
1	Periplasmic	2.08	3.49	26.0	81.12	5.8	1,025.0	1,025.0	1,599.9	3.13
	shock fluid									
	Shocked	68.40	115.00	13.5	1,520.64	109.0	1,018.6	52,272.0	52,272.0	102.00
	cell extract									
2	Whole	59.50	100.00	15.6	1,391.28	100.0	1,133.0	51,051.0	51,051.0	100.00
	cell extract									
	Periplasmic	3.64	6.50	25.0	136.50	6.0	1,328.6	3,627.0	3,627.0	4.80
	shock fluid									
	Shocked	59.50	106.00	24.6	2,193.00	96.6	1,622.8	72,420.0	72,420.0	96.60
	cell extract									
	Whole	55.90	100.00	20.7	2,269.50	100.0	1,367.4	74,970.0	74,970.0	100.00
	cell extract									

^aActivity of the whole cell extract is considered to be 100%.

released to the same degree as glucose-6-P dehydrogenase might suggest that the enzymes are located in the same fraction of the cell. Nevertheless, it is fairly obvious that, in P. putida, asparaginase is not a typical, easily released periplasmic enzyme.

Distribution of Alkaline Phosphatase

Another set of experiments was performed in order to determine the distribution of alkaline phosphatase and the percent of the enzyme released during the shock procedure. Growth in low phosphate should alter the outer membrane and facilitate release of periplasmic contents (5). Results from glucose-6-P dehydrogenase and asparaginase assays of these experiments (Table 6) are, basically, in agreement with those of Table 5. However, in these cells, asparaginase was released to a greater extent than glucose-6-P dehydrogenase; possibly as a result of the weakened outer membrane. Computation of the total alkaline phosphatase in various fractions gave unexpected results. Reportedly (5), alkaline phosphatase is released into the medium during growth. Alkaline phosphatase activity was not detected in the spent growth medium, nor in the concentrated periplasmic shock fluid. All of the activity was found in the two cell extracts (Figure 4). This suggests that in P. putida, alkaline phosphatase is less readily released than other periplasmic proteins.

TABLE 6

TOTAL CELL ALKALINE PHOSPHATASE, ASPARAGINASE, AND GLUCOSE-6-P DEHYDROGENASE
IN P. PUTIDA -- GROWN IN PHOSPHATE-DEFICIENT MEDIUM

Exp	Fraction	Percent of Total Protein	Glucose-6-P Dehydrogenase		Asparaginase		Alkaline Phosphatase	
			Total Units	Percent of Total Act. ^a	Total Units	Percent of Total Act. ^a	Total Units	Percent of Total Act. ^a
1	Periplasmic shock fluid	2.8	18.2	1.05	1,794.0	3.2	0.0	0.0
	Shocked cell extract	122.0	1,812.0	104.0	32,400.0	58.1	34.8	70.7
	Whole cell extract		1,740.0		55,800.0		49.2	
2	Periplasmic shock fluid	1.3	17.4	1.50	1,710.0	3.7	0.0	0.0
	Shocked cell extract	73.3	846.0	72.30	23,400.0	50.0	7.5	71.4
	Whole cell extract		1,170.0		46,800.0		10.5	

^aActivity of the whole cell extract was considered to be 100%.

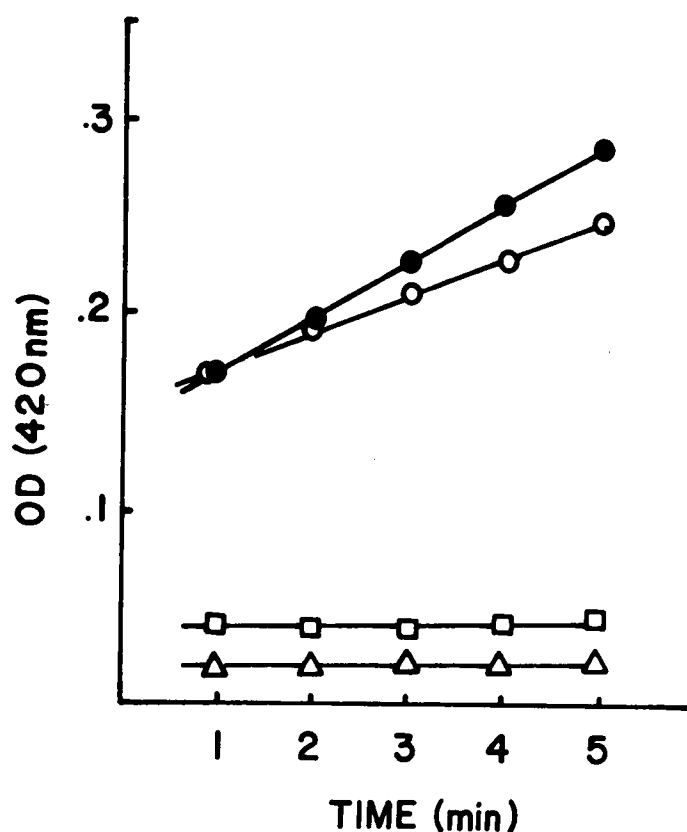


Figure 4. Alkaline phosphatase activity in cell fractions of *Pseudomonas putida*. Cells were grown in a phosphate-deficient medium (5) and glucose. Half of the culture was shocked with 0.03 M potassium phosphate buffer followed by cold distilled water with 0.01 M Mg^{++} . This yields the periplasmic shock fluid. The other half of the culture was left refrigerated. Both cell cultures were disrupted by a French Pressure Cell to produce the cell extracts.

△—△ spent growth medium.

□—□ periplasmic shock fluid.

○—○ shocked cell extract.

●—● whole cell extract.

Osmotic Shock Using Tris Buffers at High Concentrations

Shock methods described throughout this paper resulted in release of only a small amount of protein activity labelled as periplasmic enzyme. Therefore, more strenuous conditions (high concentrations of Tris buffer) were used to attempt release of a higher percentage of total asparaginase. In these experiments, retention of viability was not a main concern as long as cells remained structurally intact. This approach was also an attempt to clearly separate release of asparaginase from glucose-6-phosphate dehydrogenase.

One gram of cells that had been growing in minimal medium and glucose were washed three times with 0.03 M Tris pH 8.0. After centrifugation, the pellet was osmotically shocked by suspension in 20% sucrose-0.1 M Tris pH 8.0, followed by a 10 minute incubation and centrifugation. The pellet was rapidly resuspended in ice-cold distilled water with magnesium added to 0.1 mM. Cells were fractionated as described previously. Cell fractions were tested for glucose-6-P dehydrogenase and asparaginase activities and protein content.

Assuming activity of the whole cell extract to be 100%, it was found that about 12% of total protein was released into the periplasmic shock fluid. Thirteen to 14% of both the total glucose-6-P dehydrogenase and asparaginase activities were also detected in this fraction.

Eighty-nine to 95% of total cell protein, glucose-6-P dehydrogenase and asparaginase activities remained associated with the cell extract from shocked cells. Again, these data show that asparaginase and glucose-6-P dehydrogenase are released to the same degree, substantiating that the requirements for releasing these two proteins are very similar.

Preparation of Spheroplasts

Attempts were made to prepare spheroplasts from P. putida in order to distinguish between release of two enzymes from the cell: the periplasmic enzyme, asparaginase and the cytoplasmic marker, glucose-6-P dehydrogenase. Modifications of the Hoshino-Kageyama method (14) were used, as detailed in Table 7. The published procedure was followed in experiment 1. Glucose-6-P dehydrogenase and asparaginase activities were not detected in any of the four experimental spheroplast medium supernatant fluids, although the total protein values were in the range of 6-15 milligrams. It was thought that possibly the high sucrose content of the spheroplast medium supernatant inhibited activity; but spheroplast medium supernatant that had been dialyzed against phosphate buffer and ultrafiltered still showed no enzyme activity.

Although the supernatant fluids of the spheroplast lysate contained less protein (1.5-4.0 mg) than the spheroplast medium, activity of both enzymes was observed.

TABLE 7

PREPARATION OF SPHEROPLASTS OF P. PUTIDA^a

Fraction	Exp.	Cell Harvest Grams (Wet Wt.)	Total Volume (ml)	Protein Conc. mg/ml	Total Protein (mg)	Glucose-6-P		Asparaginase	
						Specific Act. Units/mg	Total Units	Specific Act. Units/mg	Total Units
Spheroplast	1	0.300	50	0.04	2.0	15.2	45.6	N.D. ^b	--
Lysate	2	0.305	26	0.05	1.3	28.4	55.4	560.0	546.0
Supernatant	3	0.325	26	0.10	2.6	2.4	9.4	920.0	1,794.0
	4	0.298	26	0.16	4.2	16.4	102.2	1,350.0	4,212.0

^aModifications of procedure:

EXP 1 - published procedure was followed.

EXP 2 - spheroplasts prepared with 0.75M sucrose in 0.01M potassium phosphate buffer pH 7, 0.01M MgSO₄, 0.5 mg/ml lysozyme and 0.001M EDTA; followed by a 10 minute incubation in an ice bath warmed to 30° C. Lysis step used 0.01M potassium phosphate buffer pH 6.8, 1 mM MgSO₄, 20 µg/ml DNase at 25° C for 30 minutes.

EXP 3 - same as above except that in lysis step phosphate was omitted; distilled water was substituted with more vigorous shaking at 25° C for 30 minutes.

EXP 4 - same as (3) except that in lysis step the 30 minute incubation was in an ice bath.

^bN.D. = Not determined.

The discrepancy in the distribution of protein indicates that true spheroplasts were probably not being formed. This fact, along with the intention of distinguishing release of the marker enzymes prompted the use of modified techniques. In each subsequent experiment, more severe methods of spheroplast preparation and/or lysis were employed (for example, the addition of EDTA to 1 mM in the spheroplast preparation step of experiments 2-4, and increasingly vigorous lysis methods). Incorporation of EDTA into the procedure caused an approximate 7-1/2-fold increase in the detectable asparaginase activity. The difference in experiments 2 and 3 (Table 7) was the substitution of distilled water for phosphate buffer as the diluent for spheroplast lysis. This substitution produced a two-fold increase in protein concentration and a comparable increase in asparaginase activity. Glucose-6-P dehydrogenase activity decreased rather dramatically. The latter case was the only instance where appreciable amounts of asparaginase were released without glucose-6-P dehydrogenase.

Introduction of an incubation step in the cold to increase spheroplast lysis was successful in raising the protein concentration and asparaginase activity in the spheroplast lysate supernatant of experiment 4. The glucose-6-P dehydrogenase activity was also fairly high in the lysate. Apparently conditions of this last experiment came closest to the production of true spheroplasts.

The previous three experiments probably produced only a relatively few osmotically sensitive cells. The summary of these results remains consistent with the findings of the shock experiments; that is, that asparaginase tends to be released to the same degree as glucose-6-P dehydrogenase.

CHAPTER IV

DISCUSSION

It is apparent that the extraction of periplasmic proteins from Pseudomonas species is not as routine and predictable as with the enteric rods. A more satisfactory shock procedure for P. aeruginosa might involve a combination of the Neu and Heppel and 0.2 M Mg^{++} procedures. Most periplasmic proteins would probably be released by the former method, but some of the periplasmic proteins, like alkaline phosphatase, which are believed to be linked to the exocyttoplasmic region through divalent cationic (probably Mg^{++}) bridges (5), require a high concentration of this cation for release. However, in a recent article, Ryan, Gourlie, and Tilton (24) reported that release of the periplasmic enzyme, rhodanese, from Pseudomonas aeruginosa is inhibited by either pre-incubating cells with 0.01-0.1 M Mg^{++} or by incorporation of Mg^{++} in the shock buffer; 95% of the enzyme is extracted merely by rapid suspension in cold 0.01 M Tris-HCl buffer, pH 8.4.

These conflicting facts may reflect the structural differences of the Pseudomonas envelope compared to other gram negative rods. Among these differences is the finding of Hancock and Nikaido (12), which indicates that the proteinaceous hydrophillic pores of the outer membrane of P. aeruginosa allows the passage of hydrophillic molecules

larger than those of E. coli (i.e., those of 9,000 daltons instead of 600 daltons). A very interesting difference is the fact that Pseudomonas has relatively low amounts of the lipoprotein which binds the peptidoglycan to the outer membrane (6, 19). This may be significant in terms of the extractability of periplasmic proteins. In order to gain structural support, perhaps the periplasmic proteins of Pseudomonas are more tightly bound to a cell envelope component than those of the enterics. This would compensate for the smaller amounts of the anchoring lipoprotein. If this is the case, then it could be the periplasmic proteins which form the EDTA-releasable lipopolysaccharide-glycoprotein complexes (6, 19). It would explain the unique EDTA-sensitivity of Pseudomonas species. These premises provide an explanation for the low amounts of periplasmic proteins released and the lack of binding activity. Other investigators have also had difficulty with extraction of periplasmic proteins. In a 1979 abstract, Eisenberg and Phibbs (10) reported their results with a periplasmic mannitol binding protein of P. aeruginosa. They stated that this inducible active transport system is only partially sensitive to osmotic shock. In a more recent personal communication, Eisenberg reported that in order to obtain mannitol binding protein, more severe conditions were required (i.e., the production of spheroplasts by the Hoshino-Kageyama method of treatment with sucrose,

lysozyme and a series of cold shocks). Again, these cold shocks were essential for success with Pseudomonas and were considered far superior to the $MgCl_2$ extraction method of Stinson, et al. (25).

Hoshino and Kageyama (14) described a transport system for branched chain amino acids in membrane vesicles of P. aeruginosa. In fact, they state that their membrane vesicles possess the transport activities for most amino acids. If most transport systems are completely membrane-bound, this would explain the lack of amino acid binding activity in the periplasmic shock fluid.

As yet, no proven explanation can be given for the lack of binding activity in the membrane fractions. Binding activity was expected to be membrane-associated. Preliminary results show an equal distribution of glucose dehydrogenase in all four membrane fractions although the marker, succinate dehydrogenase activity was concentrated in the inner membrane, as expected. This may support Ingram's and Mahmoudides' contention that glucose dehydrogenase is periplasmic. In a recent abstract (17), these authors reported that at least 50% of glucose dehydrogenase activity may be released from P. aeruginosa by 0.2 M $MgCl_2$ ionic shock. This release occurs when cells are grown in a medium with a high glucose concentration. Under these conditions (relatively high glucose molarity), glucose dehydrogenase activity is maximal and

one might expect repression of glucose binding proteins. The latter concept agrees with the conclusions of Whiting, Midgley and Dawes (28). That is, under high concentrations of glucose, the low affinity gluconate transport system is operative, whereas under a glucose limitation, the high affinity glucose transport system (mediated by glucose binding proteins) predominates.

From the combined data of Ingram et al. (17) and Dawes et al. (28), I hypothesize three possible conditions of glucose metabolism presented to a Pseudomonas cell. First, glucose is present in high concentrations (more than 15 mM). Under these conditions the organism produces glucose dehydrogenase and actually transports gluconate into the cell. This is the low affinity transport system of Dawes, and one would expect large quantities of glucose dehydrogenase in the cell envelope. Alternatively, cells have a limited amount of glucose available (6-15 mM). This limitation induces the production of the high affinity transport components (i.e., glucose binding proteins). A small amount of glucose (more than 6 mM) is necessary for the induction. A third condition is where glucose is in low amounts in the medium (less than 6 mM) or entirely absent when cells are growing on other nutrients (e.g., amino acids or dicarboxylic acids). Under these conditions, neither glucose dehydrogenase nor glucose binding proteins are produced and no utilization of glucose can occur.

This may explain not only the report of relatively low glucose dehydrogenase activity, but also, in our hands, the lack of binding activity in the membrane fractions, since cells intended for membrane separation were grown in a glucose-free medium.

Our medium contains a high concentration of glucose (20 mM) which by the above hypothesis would explain the lack of glucose binding activity in the shock fluids. Cells were routinely grown in minimal medium with high glucose levels since this was the prescribed method of Stinson et al. (25). Therefore, production of glucose binding proteins would be repressed. Stinson et al. (25) probably obtained activity because very large quantities of cells were shocked.

The results with Pseudomonas putida are in basic agreement with those of P. aeruginosa. Periplasmic proteins are not easily extracted and binding activity cannot be detected using the standard shock procedure. However, at present, the optimum shock procedure for P. putida requires the use of 20% sucrose in 0.01 M phosphate buffer, pH 8.3 followed by 0.01 M Mg⁺⁺ in distilled water. This gives relatively high cell viability, increased amounts of protein and asparaginase activity, but minimal amounts of glucose-6-phosphate dehydrogenase activity (Table 4, page 31). A more efficient procedure is still needed because asparaginase and alkaline phosphatase remain associated

with the cell after the above treatment. The unexpected alkaline phosphatase results (i.e., inability to detect alkaline phosphatase activity in spent growth medium and the presence of activity only in the cellular extracts) cannot, as yet, be explained. However, this phenomenon is reproducible and occurs only when cells are grown in a phosphate-deficient medium. The production of true spheroplasts should aid in localizing this enzyme in P. putida.

Although spheroplasts could not be obtained with the Hoshino-Kageyama technique, the results show that preparation of P. putida spheroplasts requires a more vigorous method than that for P. aeruginosa. Hartline, Edwards and Sando (9) have very recently published a procedure for spheroplast production in P. putida. Essentially, this method differs from the P. aeruginosa method (Hoshino-Kageyama) (14) in two ways: (1) the employment of homogenization and (2) the use of high EDTA concentrations. The fact that fairly harsh treatment is required for spheroplast formation and that difficulty is encountered when attempting to shock periplasmic proteins from P. putida by conventional methods, would indicate that these proteins are even more tightly cell-bound in P. putida than in P. aeruginosa.

One possible approach then would be the preparation of P. putida spheroplasts utilizing the method of Hartline

et al. (9). If alkaline phosphatase and asparaginase activities are again detected only in the spheroplast lysate, we would be forced to consider the possibility that these two enzymes are not periplasmic in P. putida, but cytoplasmic or tightly bound to the inside of the cytoplasmic membrane.

LITERATURE CITED

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1. Bhatti, A. R., I. W. DeVoe, and J. M. Ingram. 1976. The release and characterization of some periplasm-located enzymes of Pseudomonas aeruginosa. Can. J. Microbiol. 22: 1425-1429.
2. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
3. Cascieri, T., Jr., and M. F. Mallette. 1976. Peptide utilization by Pseudomonas putida and Pseudomonas maltophilia. J. Gen. Microbiol. 92: 283-295.
4. Chaney, A. L. and E. P. Marbach. 1962. Determination of ammonia using the Berthelot color reaction. Clinical Chem. 8: 131-136.
5. Cheng, K. J., J. M. Ingram, and J. W. Costerton. 1970. Release of alkaline phosphatase from cells of Pseudomonas aeruginosa by manipulation of cation concentration and of pH. J. Bacteriol. 104: 748-53.
6. Costerton, J. W., M.R.W. Brown and J. M. Sturgess. 1979. The cell envelope: its role in infection, pp. 41-59. In Pseudomonas aeruginosa. Clinical Manifestations of Infection and Current Therapy. R. G. Doggett. Academic Press, New York.
7. Day, D. F., and J. M. Ingram. 1971. Localization of L-asparaginase in Pseudomonas aeruginosa. Can. J. Microbiol. 17: 1025-28.
8. DiRienzo, J. M., K. Nakamura, and M. Inouye. 1978. The outer membrane proteins of gram negative bacteria: biosynthesis, assembly and functions. Ann. Rev. Biochem. 47: 481-532.
9. Edwards, W. V., J. J. Sando, and R. A. Hartline. 1979. Transport of C5-dicarboxylate compounds by Pseudomonas putida. J. Bacteriol. 139: 748-54.
10. Eisenberg, R. C., and P. V. Phibbs, Jr. 1979. Mannitol binding protein in Pseudomonas aeruginosa. Am. Soc. Microbiol. Ann. Meet. K 101: 162 (Abstr.).

11. Gray, G. W., and S. G. Wilkinson. 1965. The action of EDTA on Pseudomonas aeruginosa. J. Appl. Bacteriol. 28: 153-64.
12. Hancock, R.E.W., and H. Nikaido. 1978. Outer membranes of gram negative bacteria. XIX. Isolation from Pseudomonas aeruginosa PAO 1 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. 136: 381-90.
13. Hauge, J. G. 1966. Glucose dehydrogenases-particulate I. Pseudomonas species and Bacterium anitratum, pp. 92-98. In Methods of Enzymology, Vol. 9. S. P. Colowick and N. O. Kaplan (ed.). Academic Press, New York.
14. Hoshino, T. and M. Kageyama. 1979. Sodium-dependent transport of L-leucine in membrane vesicles prepared from Pseudomonas aeruginosa. J. Bacteriol. 137: 73-81.
15. Kasahara, M., and Y. Anraku. 1974. Succinate dehydrogenase of E. coli. Membrane vesicle activation and properties of the enzyme. J. Biochem. 76: 959-66.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-75.
17. Mahmoudides, G., and J. M. Ingram. 1978. The cyclical appearance of glucose dehydrogenase in Pseudomonas aeruginosa. Am. Soc. Microbiol. Ann. Meet. K III: 145 (Abstr.).
18. Malamy, M. H., and B. L. Horecker. 1964. Release of alkaline phosphatase from cells of E. coli upon lysozyme spheroplast formation. Biochem. 3: 1889-93.
19. Meadow, P. 1975. Wall and membrane structures in the genus Pseudomonas, pp. 67-98. In Genetics and Biochemistry of Pseudomonas. P. H. Clarke and M. H. Richmond (ed.). John Wiley and Sons, London.
20. Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from Escherichia coli by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. 240: 3685-92.

21. Oxender, D. L. 1974. Membrane transport proteins. Ch. 2, pp. 25-79. In Biomembranes v. 5. L. A. Mansen (ed.). Plenum Press, New York.
22. Oxender, D. L., and S. Quay. 1977. Binding proteins and membrane transport. Ann. N. Y. Acad. Sci. pp. 358-71.
23. Reber, G., M. Belet, and J. Deshusses. 1977. Myo-inositol transport system in Pseudomonas putida. J. Bacteriol. 131: 872-75.
24. Ryan, R. W., M. P. Gourlie, and R. C. Tilton. 1979. Release of rhodanese from Pseudomonas aeruginosa by cold shock and its localization within the cell. Can. J. Microbiol. 25: 340-51.
25. Stinson, M. W., M. A. Cohen, and J. M. Merrick. 1976. Isolation of dicarboxylic acid and glucose binding proteins from Pseudomonas aeruginosa. J. Bacteriol. 128: 573-79.
26. Stinson, M. W., M. A. Cohen, and J. M. Merrick. 1977. Purification and properties of the periplasmic glucose binding protein of Pseudomonas aeruginosa. J. Bacteriol. 131: 672-81.
27. Torriani, A. 1960. Influence of inorganic phosphate in the formation of phosphatases. Biochim. Biophys. ACTA. 38: 460-69.
28. Whiting, P. H., M. Bidgeley, and E. A. Dawes. 1976. The regulation of transport of glucose, gluconate, and 2-oxogluconate and of glucose catabolism in Pseudomonas aeruginosa. Biochem. J. 154: 659-68.
29. Yellin, T., and J. Wriston. 1966. Purification and properties of guinea pig asparaginase. Biochem. 5: 1605-12.

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