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A STUDY OF THE MANNITOL BINDING PROTEIN ACTIVE TRANSPORT
SYSTEM AND A CATABOLITE REPRESSION RESISTANT MUTANT
IN PSEUDOMONAS AERUGINOSA

by

Joseph Arthur Wolff

A Thesis
Submitted to the
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in partial fulfillment of the
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Western Michigan University
Kalamazoo, Michigan
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A STUDY OF THE MANNITOL BINDING PROTEIN ACTIVE TRANSPORT
SYSTEM AND A CATABOLITE REPRESSION RESISTANT MUTANT
IN PSEUDOMONAS AERUGINOSA

Joseph Arthur Wolff, M.S.

Western Michigan University, 1984

Cold-shock extraction procedures were used for the efficient release of mannitol binding protein. Magnesium chloride and cold-shock released 92% of the total whole cell mannitol binding protein activity without release of detectable cytoplasmic marker protein glucose-6-phosphate dehydrogenase. Arsenate inhibited mannitol uptake to the same extent as azide. The simultaneous loss and gain of mannitol binding protein activity with mannitol uptake activity in mutants and revertants respectively, was consistent with periplasmic mannitol binding protein as a required component of mannitol uptake. A catabolite repression resistant mutant for mannitol uptake was isolated and described. This mutant, designated WEP-1, escaped catabolite repression of mannitol uptake in the presence of succinate. Downstream inducible enzymes of carbohydrate catabolism did not escape succinate repression to the same extent as did the early proteins required for mannitol uptake. This appears to be the first catabolite repression resistant mutant described for Pseudomonas.

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Joseph Arthur Wolff

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WESTERN MICHIGAN UNIVERSITY

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INTRODUCTION

Transport

The study of biological transport systems, in particular bacterial transport systems, involves understanding the processes by which a cell is able to translocate molecules across cellular membranes. Biological transport systems may involve several distinct mechanisms commonly referred to as facilitated diffusion, active transport and group translocation (Boos, 1974; Saier, 1977; Wilson, 1978).

Facilitated diffusion is a passive event with respect to energy utilization in which a solute molecule is transported down a concentration gradient as it moves across the membrane. This type of transport differs from random diffusion in that a protein carrier located proximal to the membrane is believed to facilitate or hasten the establishment of the solute concentration equilibrium across the membrane. Also, the initial rate of transport is saturable. No membrane carrier exists in random diffusion and the initial rate of transport is always dependent on the concentration gradient.

Active transport also involves a protein carrier. However, this mechanism includes an energy utilizing event which allows for the transport of solute molecules up a concentration gradient. Both of these carrier mediated systems involve specific protein components that transport highly stereospecific compounds or their closely related analogues.

The group translocation mechanism resembles active transport.

However, in this case the solute molecule is chemically modified as it crosses the membrane and no longer exists as the same chemical structure present before transport.

In bacteria, three major types of transport systems operate: the group translocation system, the membrane bound transport system, and the binding protein transport system (Wilson, 1978). All of these systems share features common to active transport, although in strict terms, the group translocation system is not active transport. These transport systems display saturable transport kinetics, require energy to concentrate against a solute gradient, and are physiologically vectorial events with respect to solute translocation across the cell membrane.

Conversely, many differences between these transport systems may be found. Perhaps the most characteristic difference arises when comparing energy coupling events. The group translocation system uses phosphoenolpyruvate as an energy-phosphate donor which chemically modifies the translocated solute molecule as it enters the cell. Hence, this system cannot be considered true active transport (Saier, 1977). The membrane bound transport system requires an electrochemical gradient (proton motive force) in order to transport molecules either directly or indirectly via symport/antiport mechanisms (Berger & Heppel, 1974; Kaback, 1972; Klein & Boyer, 1972). Finally, the binding protein transport system requires an adenosine triphosphate (ATP) or other similar transferase substrate energy source in order to function (Berger, 1973; Berger & Heppel, 1974; Ferenci, Boos, Schwartz & Szmelcman, 1977; Romano, Voytek & Bruskin, 1980).

The mechanisms of solute translocation and energy transduction in the binding protein transport system have been the object of considerable study within the last decade. Numerous binding proteins have been isolated and described. Examples include binding proteins specific for the groups of related amino acids lysine, arginine, and ornithine (Rosen, 1971); and leucine, isoleucine, and valine in Escherichia coli (Piperino & Oxender, 1966) and in Pseudomonas aeruginosa (Hoshino & Kageyama, 1980). Numerous examples of binding proteins specific for individual amino acids can also be found: arginine (Rosen, 1973), histadine (Rosen & Vasington, 1971; Lever, 1972), cysteine (Berger & Heppel, 1972), glutamine (Weiner & Heppel, 1971), leucine (Furlong, 1970). Binding proteins specific for the ions phosphate (Medveczky & Rosenberg, 1969), and sulfate (Pardee, Prestidge, Whipple & Dreyfuss, 1966) have been reported. A binding protein specific for the vitamin thiamine and its mono- and diphosphate derivatives (Nishimune & Hayashi, 1971) was reported as well as a binding protein specific for the vitamin cyanocobalamine (Taylor, Norrell & Hanna, 1972). Carbohydrate specific binding proteins include those for arabinose (Parsons & Hogg, 1974), glucose (Stinson, Cohen & Merrick, 1976), galactose (Anraku, 1968a, 1968b), ribose (Aksamit & Koshland, 1972), maltose (Kellermann & Szmelcman, 1974), and mannitol (Eisenberg & Phibbs, 1982).

Generally, binding proteins refer to small molecular weight proteins (typically 30,000 daltons) with highly stereospecific active sites that reversibly bind ligand substrate without altering the ligand's chemical structure (Oxender & Quay, 1976). Although binding

proteins interact briefly with the ligand molecules and display kinetic activity similar to enzymes, because the chemical structure of the interacting ligand is not altered, binding proteins are not considered enzymes.

Binding protein transport systems are not phenomena strictly confined to lower organisms (Wasserman, Corradino & Taylor, 1968; Wilson, 1978). However, in bacteria they are found exclusively in the Gram negative classification.

Binding proteins have been shown to be a constituent of the periplasmic space, a region between the inner and outer membrane of Gram negative bacteria. This periplasmic association was determined in part by the use of mild osmotic shock procedures that selectively disrupt the outer membrane of E. coli while leaving the inner membrane intact (Neu & Heppel, 1965). Osmotically shocked E. coli releases periplasmic proteins into the surrounding buffer medium while retaining cell viability. The binding protein, which is water soluble, may then be detected or recovered from the shock fluid along with other soluble periplasmic proteins. The osmotic shock treatment in E. coli also causes a loss in binding protein transport activity (Oxender & Quay, 1976; Wilson & Holden, 1969).

This relationship between the location and function of binding proteins is further illustrated by the study of transport in membrane vesicles. Membrane vesicles prepared from Gram negative bacteria by the methods of Kaback (1972) are devoid of outer membrane and cytoplasmic components, retain membrane bound transport functions for amino acids and carbohydrates but lose binding proteins and their

associated transport functions (Gordon, Lombardi & Kaback, 1972).

This is also true of vesicles prepared from P. aeruginosa by the method of Stinnett, Guymon, and Eagon (1973).

Binding protein transport systems generally exhibit kinetic evidence to suggest that the binding protein is the rate limiting step in the transport process (Oxender & Quay, 1976). While this may be true, it is believed that binding proteins are not directly responsible for the specific translocation event of moving solute molecules across the inner membrane into the bacterial cell. Rather, binding proteins are believed to interact with other tightly bound or intrinsic components of the inner membrane (Ames & Lever, 1970; Ames & Spudich, 1976). It has not always been possible to obtain direct physical evidence supporting the existence of these intrinsic membrane components. Instead, researchers have had to rely on genetic evidence to define the role these components play in transport. In the case of histidine transport in Salmonella typhimurium, it was shown that two membrane bound gene products were obligatory for histidine transport, and by using newly developed electrophoretic techniques, Ames and Nikaido (1978) were able to isolate these membrane components and, thus, gain direct biochemical evidence substantiating genetic data concerning their existence.

In addition to the transport phenomena associated with binding proteins, these gene products also function in the chemotactic response. Chemotaxis is the ability of a motile organism to selectively move up or down a chemical concentration gradient. In bacteria, binding proteins appear to be necessary for the chemotactic response

to occur in chemical gradients for which the binding protein also serves in transport (Adler, 1975). However, while a binding protein may be obligatory for chemotaxis to occur, it appears that chemotaxis is not dependent on the transport event itself. Apparently, transport and chemotaxis are both mediated by binding protein interactions at separate receptor sites on the cell membrane which are specific for either transport or chemotaxis (Adler, 1969).

Catabolite Repression

Catabolite repression is a term first coined by Magasanik (1961) which he used to explain the "glucose effect," a phenomenon whereby glucose specifically inhibits the induction of synthesis of some other non-glucose metabolizing enzymes. The glucose effect was first demonstrated in E. coli by Monod (1947) who found that cultures showed biphasic growth and preferentially utilized glucose over lactose when grown in the presence of both sugars. Magasanik believed that the build-up of catabolic products or intermediates derived from the kinetically favored utilization of glucose caused the repression of the synthesis of enzymes otherwise induced by lactose.

The key to understanding this problem came much later when Pastan and Perlman (1970) were able to show that exogenous cyclic adenosine 3',5'-monophosphate (c-AMP) could relieve the glucose controlled repression of β -galactosidase synthesis in cells which were induced for the expression of the lactose operon (i.e., c-AMP abolished the glucose effect). A broad based understanding of the control mechanisms for the glucose effect in E. coli and related bacteria has since

evolved.

Briefly, the uptake of glucose inhibits the activity of the enzyme adenylcyclase which catalyzes the reaction producing c-AMP. Cyclic-AMP is a positive co-factor to a binding protein (CAP) which, in the presence of c-AMP, is able to bind to a site on the DNA promoter region of the lactose operon. This in turn increases the efficiency of the RNA polymerase binding to the promoter region thus permitting expression of the operon.

In P. aeruginosa the glucose effect is not observed. Instead, when this organism is presented with two energy sources, one a tri-carboxylic acid cycle (TCA) intermediate such as succinate, malate, or citrate, and a carbohydrate energy source such as glucose or mannitol, the order of diauxic utilization is the reverse of E. coli: biphasic growth occurs and glucose catabolic enzymes are repressed until TCA intermediates are consumed (Tiwari & Campbell, 1969). This basic difference in glucose utilization between E. coli and P. aeruginosa may be rationalized by considering the different catabolic pathways. In E. coli energy is obtained when glucose is fermented by the Embden-Meyerhof pathway. However, in P. aeruginosa, where energy is obtained strictly by oxidative phosphorylation, glucose is first catabolized by the Entner-Doudoroff pathway which then feeds intermediates into the TCA cycle. The preferential utilization of TCA cycle intermediates circumvents the Entner-Doudoroff pathway and allows for a more direct and rapid acquisition of carbon and energy.

The mechanism of catabolite repression in P. aeruginosa is not understood. Smyth and Clarke (1975) reported that exogenous c-AMP

could partially relieve the catabolite repression of amidase enzyme when repression was caused by lactate but not when caused by succinate. If, in fact, c-AMP has a physiologically significant role in the catabolite repression mechanism, one might expect to find endogenous c-AMP concentrations to vary according to growth condition. Shapiro, Agabin-Keshishian, Hirsch, and Rosen (1972) reported a ten-fold increase for intracellular c-AMP levels in starving cells as compared to cells growing on glucose. Siegel, Hylemon, and Phibbs (1977) extensively investigated intracellular c-AMP levels in cells utilizing six different carbon sources (including succinate and glucose) and at four phases of growth. They reported an average intracellular c-AMP value of $16 \mu\text{M}$ with no significant differences between growth conditions. Phillips and Mulfinger (1981) also found that the influence of carbon source did not significantly affect the intracellular c-AMP level. They also demonstrated that c-AMP was unable to relieve the catabolite repression of histadase synthesis as caused by either succinate or lactate. The results of these reports suggest that c-AMP is probably not involved in the mechanisms which control catabolite repression in P. aeruginosa to the same extent or in the same straight forward manner that occurs in E. coli.

Mannitol Uptake in P. Aeruginosa

Mannitol utilization in P. aeruginosa results in the induction of a transport system which is highly specific for mannitol, is energy and temperature dependent, and displays saturation kinetics. All these features are common to an active transport mechanism (Eagon

& Phibbs, 1971; Phibbs & Eagon, 1970). Growth on mannitol also induces the enzymes mannitol dehydrogenase and fructokinase, whose product (fructose-6-phosphate) is then isomerized by phosphoglucose isomerase, oxidized by glucose-6-phosphate dehydrogenase, and further metabolized by the enzymes of the Entner-Doudoroff pathway (Phibbs, McCowen, Feary & Blevins, 1978). All of these enzymes, with the exception of phosphoglucose isomerase, are specifically inducible (Phibbs et al., 1978) and subject to strong catabolite repression by carboxylic acid intermediates of the Krebs Cycle (Phibbs et al., 1978; Tiwari & Campbell, 1969). Membrane vesicles prepared from cells induced by mannitol are unable to transport mannitol (Pont, 1973; Stinnet, Guymon, and Eagon (1973) and mannitol binding protein activity was detected in shock fluid prepared from such cells (Eisenberg & Phibbs, 1978, 1979, 1982; Pont, 1973).

Attempts at extracting binding proteins have favored using "shock buffer" extraction procedures (Cheng, Ingram & Costerton, 1970a, 1970b; Hoshino & Kageyama, 1980; Neu & Heppel, 1965). The use of these techniques has consequently helped support evidence concerning the periplasmic nature of binding proteins. Additionally, these methods of extraction, by nature of the concentration of proteins in the periplasm relative to the whole cell, have also allowed for some advantage in purification over whole cell extracts. Apparently, however, shock procedures do not work with equal success in all species of Gram negative bacteria. P. aeruginosa has been shown to be particularly sensitive to lysis on exposure to ethylenediamine-tetraacetate (EDTA) (Eagon & Carson, 1965; Stinnet, Gilleland & Eagon,

1973), a principal component of conventional shock buffers (Neu & Heppel, 1965). Cheng et al. (1970a, 1970b) developed a shock buffer system using 0.2 M MgCl_2 to extract alkaline phosphatase from the periplasm of P. aeruginosa and Stinson et al. (1976) modified this protocol for the isolation of a dicarboxylic acid binding protein and a glucose binding protein. Eisenberg and Phibbs (1978, 1979, 1982) used the method of Stinson to isolate and characterize the mannitol binding protein from P. aeruginosa. Mannitol binding protein extraction by this method was uncharacteristically inefficient and its putative periplasmic location and function was, therefore, unresolved.

The purpose of this study was to better establish the periplasmic location of the mannitol binding protein in Pseudomonas aeruginosa and determine if mannitol transport is, in fact, a mannitol binding protein mediated active transport system. The characterization of a catabolite repression resistant (crr) mutant for mannitol uptake was also studied since this crr mutant was isolated during the course of this study in an effort to isolate a mannitol transport constitutive mutant. It appears, from the known available literature, that this is the first instance of a crr mutant identified for Pseudomonas, and may be a significant development towards eventual elucidation of the currently unknown mechanism(s) of catabolite repression in Pseudomonas and related bacteria.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Pseudomonas aeruginosa strain PAO was cultured under the following standard conditions unless otherwise indicated. Cultures were grown in 2.8 liter Fernback flasks containing 1000 ml of synthetic minimal salts medium (50 mM potassium phosphate, 15 mM ammonium sulfate, 0.80 mM magnesium chloride, 2 mM ferrous sulfate, pH 7.0) plus indicated carbon and energy source. Cultures were incubated at 37°C in a New Brunswick gyratory shaker (250 RPM) and growth was monitored by using a Klett colorimeter with a number 66 filter. Minimal salts media and carbon and energy sources were autoclaved separately.

Mutants

Mutant strains of P. aeruginosa (wild-type strain PAO), isolated for their inability to utilize mannitol as a carbon and energy source, were made available from the laboratory of P. V. Phibbs, Jr. Strains 105 and 133 have been previously described (Phibbs et al., 1978) and were obtained using N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) as mutagen. Strains 539, 555 and 601 were obtained using ethyl methane sulfonate as mutagen. Mannitol utilizing spontaneous revertants of mutant strains were isolated as described by Phibbs et al. (1978). Revertant frequencies for all these mutants was approximately 0.2×10^{-9} .

Buffers

All buffers were made with distilled-deionized water. Buffer A was composed of 50 mM potassium phosphate, pH 7.0. CPA buffer was composed of 5 mM citrate, 10 mM potassium phosphate, and 3 mM sodium azide, pH 6.0. TMAK buffer was composed of 10 mM Tris-hydrochloride (Tris-Cl), 1 mM magnesium chloride, 3 mM sodium azide and 50 mM potassium chloride, pH 7.5.

Binding Protein Assay

A nitrocellulose membrane filtration assay method developed by Stinson et al. (1977) and modified by Eisenberg and Phibbs (1982) was used to measure mannitol binding protein (MBP) activity. Protein solutions, up to 400 µg protein per ml, were mixed with 0.5 ml 2 µm ¹⁴C-mannitol (50 mCi/mmole) in TMAK buffer and taken up in TMAK buffer to 1.0 ml final volume at room temperature. The solution was immediately filtered on a premoistened 24 mm filter (Millipore type HMAK, 0.45 µm) and washed with 3 ml TMAK buffer. The filter was removed under vacuum and transferred to a liquid scintillation vial containing 10 ml of Aquasol (New England Nuclear L.C.S. cocktail). Radioactivity was measured using a Searle Isocap/300 spectrophotometer. One unit of MBP activity is defined as one picomole (pmole) mannitol bound per filter. Specific activity is expressed as units per mg protein. All activities reported were results calculated from linear slopes obtained by plotting radioactivity bound per filter vs. protein concentration. The lower limit for detecting MBP in crude

periplasmic extracts was approximately 2 pmoles per mg protein.

Magnesium Chloride Cold-Shock Extraction

The magnesium chloride cold-shock procedure was fundamentally performed as described by Hoshino and Kageyama (1980). Cell cultures were grown to mid-exponential phase under standard conditions using 30 mM lactate plus 10 mM mannitol as carbon and energy sources. Cells were harvested at 10,000 x g for 10 minutes and washed once with buffer A, then resuspended in 0.2 M MgCl_2 plus 50 mM Tris-Cl, pH 7.3 at approximately 0.1 to 0.2 g wet weight per ml. Resuspended cells were incubated at 30°C for 10 minutes, then rapidly chilled in an ice water bath for 15 minutes. Chilled cells were then rewarmed to 30°C and incubated for another 10 minutes and rechilled for 15 minutes. Shocked cell suspensions were centrifuged (27,000 x g for 10 min at 4°C) and collected supernatant was dialyzed against CPA buffer (4°C) to reduce MgCl_2 concentration to less than 0.1 mM.

Mannitol Uptake Assay

A membrane filtration method developed by Phibbs et al. (1978) was used to determine ^{14}C -mannitol uptake in whole cells. Cultures were harvested by centrifugation (10,000 x g for 10 min at 23°C), washed twice in an equal volume of buffer A (37°C) and resuspended with buffer A to a Klett reading (#66 filter) of 200 (ca. 0.35 mg cell protein per ml). Suspended cells were placed in a flask and kept well aerated in a gyratory shaker (37°C, 250 RPM). A sample for assay (0.175 ml) was transferred to a 50 ml flask contained in a water

bath reciprocal shaker (37°C, 120 cycles per min) and diluted with 0.45 ml buffer A (37°C). Reactions were started by the addition of 0.30 ml ^{14}C -mannitol (0.5 mM at 1.6 μCi per μmole to give a final concentration of 0.1 mM at 3000 cpm per nmole). Samples (0.1 ml) were removed at one minute intervals (for up to five minutes) and diluted into 2.0 ml of buffer A (37°C) which overlayed a membrane filter (0.45 μm pore size). Diluted samples were immediately filtered and washed with 10 ml of buffer A at 37°C, twice. Washed filters were removed under vacuum and transferred directly into a liquid scintillation vial containing 10 ml of Aquasol. Radioactivity was measured using a Searle Isocap/300 spectrophotometer. Uptake activity is expressed as nmole mannitol per mg cell protein.

Enzyme Assays

Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49, G-6-PDH) activity was determined by the method of Hylemon and Phibbs (1972) using a 1.0 ml reaction mixture (pH 8.0) containing 10 mM MgCl_2 , 0.3 mM NADP, 2 mM glucose-6-phosphate, 50 mM Tris-Cl and 5 mM 2-mercaptoethanol. Mannitol dehydrogenase (E.C.1.1.1.67) activity was assayed by the method of Siegel et al. (1977) using a 1.0 ml reaction mixture (pH 8.0) containing 0.3 mM NAD, 42 mM mannitol, 50 mM Tris-Cl and 5 mM 2-mercaptoethanol. Fructokinase (E.C.2.7.1.4) activity was determined by the method of Phibbs et al. (1978) using a 1.0 ml reaction mixture (pH 8.0) containing 5 mM MgCl_2 , 0.25 mM NADP, 5 mM $\text{ATP}(\text{Na}^+)$, 10 mM fructose, 0.2 International Units (I.U.) of commercial phosphoglucoisomerase, 0.36 I.U. of commercial glucose-6-phosphate dehydro-

genase and 50 mM Tris-Cl. The 6-phosphogluconate dehydratase (E.C.4.2.14) and 2-keto-3-deoxy-6-phosphogluconate aldolase (E.C.4.1.2.14) combined activities were assayed by the method of Hylemon and Phibbs (1972) using a 0.5 ml reaction mixture (pH 8.0) containing 0.23 mM NADPH, 8 mM 6-phosphogluconate, 7.6 I.U. of commercial lactate dehydrogenase, and 31 mM imidazole. Enzyme activity was determined on a Gilford recording spectrophotometer at room temperature (ca. 23°C). Enzyme reactions were substrate dependent and initial velocity was proportional to the protein extract concentration. One unit of enzyme activity is defined as 1 μ mole NAD(P) either reduced or oxidized per minute. Specific activity is expressed as units per μ g protein. All activities reported were calculated from initial velocities obtained by titrating samples.

Lysozyme Cold-Shock Cell Fractionation

Spheroplasts were prepared by modifying the method of Hoshino and Kageyama (1979) developed for use in making vesicles from P. aeruginosa. Early exponential phase cells grown on 30 mM lactate plus 10 mM mannitol were harvested (10,000 x g for 10 min at 23°C), washed once with buffer A (23°C) and then suspended in a large bottomed 500 ml stainless steel beaker to a final concentration of ca. 10^{10} cell per ml with a buffer containing 10 mM potassium phosphate (pH 7.0), 0.75 M sucrose, 10 mM magnesium sulfate and 0.5 mg lysozyme per ml. This suspension was then placed in an ice-water bath and gently agitated for ten minutes. The flask was then warmed to 30°C in a 37°C water bath, again with gentle mixing. This cold-

shock treatment was repeated twice before the cell suspension was incubated (30°C) with gentle shaking in a New Brunswick gyratory shaker and monitored continuously for up to thirty minutes using a Nikon phase-contrast microscope. This treatment turned cells into spheroplasts. The completion of spheroplast formation was ascertained visually by observing the change from the normal rod shaped appearance of cells to large round shaped cells (spheroplasts) as viewed under the microscope. Conversion was estimated to be 90% by visual approximation. Spheroplasts were then collected by centrifugation at 27,000 x g for 30 minutes at 4°C. The supernatant was removed, dialyzed against TMAK buffer (4°C) and frozen at -60°C until the procedures for cytoplasmic and membraneous extracts were completed. This frozen supernatant was the spheroplast supernatant. The remaining spheroplast pellet was resuspended in 60 ml TMAK buffer containing 2 µg each DNase and RNase per ml. The suspension was then homogenized in a Virtis "45" blender at 45,000 RPM for two minutes which lysed the spheroplasts. The resulting mixture was centrifuged (27,000 x g for 30 min at 4°C) and the supernatant collected (cytoplasm fraction). The remaining pellet was washed once with TMAK buffer, centrifuged (3,000 x g for 20 min at 4°C) to sediment remaining whole cells, and this supernatant again centrifuged at 27,000 x g for 30 minutes at 4°C. This final pellet was retained as the membrane fraction. The membrane fraction was suspended up to 20 ml with TMAK buffer and frozen at -60°C. Cytoplasmic extract and spheroplast supernatant were dialyzed against TMAK buffer (4°C) before being frozen (-60°C) until a time when enzyme and binding protein assays in the various

fractions could all be assayed at the same time; this provided for ensuring that total units obtained by summing all fractions were comparable and differences in fractions compared were not due to differential inactivation or denaturation by variation in sample handling.

Catabolite Repression Resistant Mutant

Selection

A catabolite repression resistant mutant of PAO was selected by cyclicly transferring late exponential or early stationary cultures between test tubes containing 5 ml of minimal salts medium plus 50 mM succinate or 50 mM mannitol. Cultures were otherwise grown under standard conditions. Approximately 30 cycles of two complete transfers between succinate and mannitol media per cycle were used to select for mutants.

Screening

Cultures screened for the presence of catabolite repression resistant mutants were grown on 20 mM succinate to mid-exponential phase and then plated (ca. 300 colonies per plate) onto minimal salts agar (1.5% agar in minimal salts medium) plus 100 mM succinate and 50 mM ^{14}C -mannitol (1mCi/mmmole). Plates were incubated (37°C) for 48 hours or until small colonies began to appear (1-3 mm diameter) before a sterile filter paper disk was placed onto the surface of the agar allowing the colonies to uniformly adsorb to the paper. The colony imprinted paper was removed, dried at room temperature and exposed to

Kodak Blue Brand X-ray film (room temperature for 48 hours, no screen). The film was developed and analyzed for mutant isolation.

Isolation

Exposed and developed X-ray film used in mutant screening exhibited darkened spots of various densities which were coincidental with the pattern of colonies adsorbed onto the filter paper. Given that all colony adsorption to filter paper was uniform (i.e., all colonies tended to adsorb at approximately equal mass per area), those colonies which caused dark spots on film exposed to filter paper contained more ^{14}C (labeled mannitol) than colonies which resulted in light spots. Hence, dark spot colonies were assumed to be capable of mannitol uptake in the presence of succinate and were picked as putative mutants for mannitol uptake. Individual mutant colonies were purified by streak plating onto nutrient agar medium and these isolated colonies were then restreaked on ^{14}C -mannitol screening medium alongside the parent PAO strain. Again, colony filter paper imprints exposed to X-ray film were used to verify mutant isolation.

The method described here for the screening and isolation of mannitol transport mutants of PAO was adapted from a technique originally described by Zwaig and Lin (1966) for isolation of Escherichia coli mutants resistant to catabolite repression.

Other Assays

Protein was measured by either the method of Lowry, Rosebrough, Farr, and Randall (1951) using crystalline bovine serum albumin as a

standard or by the U.V. spectrophotometric methods of Kalb and Bernlohr (1977).

Chemicals

D-(1-¹⁴C)-mannitol (50 mCi/mmol) was purchased from New England Nuclear. All other reagents were either analytical or reagent grade quality obtained from the usual commercial sources.

RESULTS

Localization of Mannitol Binding Protein

Lysozyme Cold-Shock Extraction

Periplasmic, cytoplasmic and membranous extracts were prepared from cells by the lysozyme cold-shock protocol described in Materials and Methods. Extracts were assayed for mannitol binding protein (MBP) activity and glucose-6-phosphate dehydrogenase (G6PDH) activity and results are indicated in Table 1. Assays were performed with and without 10 mM 2-mercaptoethanol and no change in activities was evident. Extracts were also assayed for activity with and without 0.1% (v/v) Triton X-100 detergent. In the presence of Triton X-100 all mannitol binding protein activity appeared to be inhibited although Triton X-100 had no effect on the G6PDH activity. Data in Table 1 show that MBP was not present in the membrane fraction and distribution of G6PDH and MBP in spheroplast supernatant and cytoplasm fractions is best explained on the basis of G6PDH as a cytoplasmic enzyme and MBP as a periplasmic protein.

Magnesium Chloride Cold-Shock Extraction

The magnesium chloride cold-shock extraction procedure was performed as described in Materials and Methods. The remaining shocked cell pellet was frozen and thawed in order to increase cell fragility, diluted up to 10 ml with CPA buffer (0°C), and ruptured by ultrasonic

Table 1.

Distribution of Mannitol Binding Protein (MBP) Activity and Glucose-6-Phosphate Dehydrogenase (G6PDH) Activity in Cytoplasmic, Membrane, and Spheroplast Supernatant Fractions Obtained From Lysozyme Cold-Shock Treatment¹

Fraction	Volume (ml)	G6PDH (Units/ml) Total		MBP (Units/ml) Total		Percent of Total Recovered	
						G6PDH	MBP
Spheroplast Supernatant	63.0	0.079	4.98	26.90	1690	25	75
Cytoplasmic	92.5	0.161	14.90	6.14	568	75	25
Membrane	20.0	0.006	0.12	<1	<1	<1	<1

¹See Materials and Methods section for general details and text of Results section for specific details of experimental protocol.

oscillation (3 10 sec bursts with intermittent cooling of probe and sample). The ruptured sample was centrifuged ($27,000 \times g$ for 10 min at 4°C) and the supernatant was dialyzed against CPA buffer (4°C). Both the extracted shock supernatant and the extracted shock pellet were assayed for mannitol binding protein activity and glucose-6-phosphate dehydrogenase activity.

Results in Table 2 show that almost all of the mannitol binding protein activity, 92% of the total, was located in the shock supernatant fraction with only 8% of the total activity remaining in the shock pellet fraction. This is in marked contrast to the activity of the cytoplasmic marker enzyme, glucose-6-phosphate dehydrogenase, which was strictly confined (100% of total detectable activity) to the shock pellet fraction.

The cold-shock extraction of mannitol binding protein without the use of 0.2 M MgCl_2 was examined for extraction efficacy. Cells were grown and treated as described under the magnesium chloride cold-shock extraction protocol; however, magnesium chloride was not included in the 0.1 M Tris-Cl buffer (pH 7.3). The resulting cold-shocked pellet was assayed for mannitol uptake activity as previously described. Uptake in the magnesium chloride cold-shock pellet (Table 2) could not be analyzed because the cells were osmotically unstable and lysed when washed with buffer A.

The results of this experiment testing cold-shock alone are presented in Table 3. Magnesium-free cold-shock treated cells released mannitol binding protein with almost three times the specific activity of magnesium cold-shock treated cells. The total amount of

Table 2.

Distribution of Mannitol Binding Protein (MBP) Activity and
Glucose-6-Phosphate Dehydrogenase (G6PDH) Activity in
0.2 M MgCl_2 Cold-Shock Supernatant Fluid and Cell Pellet¹

Fraction	Volume (ml)	G6PDH (Units/ml) Total		MBP (Units/ml) Total		Percent of Total Recovered	
						G6PDH	MBP
Shock Supernatant	31.0	0.01	<1	17.30	536	<1	92
Shock Pellet	11.6	0.70	8.13	4.14	48	100	8

¹ See Materials and Methods section and text of Result section for specific experimental protocol.

Table 3.

Synergistic Effect of 0.2 M MgCl_2 and Cold-Shock on Release of Mannitol Binding Protein (MBP) for *P. Aeruginosa*

	Mannitol Uptake (nmole/mg-min)	MBP (Units/mg)	Protein (mg/ml)	Total MBP Released (Units/9.3 ml)
Control	34	—	—	—
Cold-Shock	3.3	271	0.062	156
Cold-Shock Plus 0.2 M MgCl_2	—	91.4	0.776	659

¹See Materials and Methods section and text of Results section for experimental details.

mannitol binding protein released in the magnesium shock treatment was four times greater than the amount released in the magnesium-free shock treatment. In addition to the highly specific release of mannitol binding protein, cold-shock treated cells exhibited a concomitant loss of mannitol uptake activity of about 90% that observed in an induced normal whole cell control.

Mutant and Revertant Analysis

Mutants unable to utilize mannitol as a carbon and energy source and spontaneous revertants which recovered the ability to utilize mannitol were assayed for mannitol uptake, mannitol binding protein, and mannitol dehydrogenase (MDH) activities. Cells were grown under standard conditions to mid-exponential phase on 30 mM lactate plus 10 mM mannitol, were harvested by centrifugation (10,000 x g for 10 min at 23°C), and washed in an equal volume of buffer A (37°C). Mannitol uptake assays were performed as previously described. Mannitol binding protein was extracted by the magnesium chloride cold-shock procedure and assayed as previously described. Whole cell pellets from harvested cultures were frozen, thawed and diluted to 10 ml with CPA buffer (0°C) before rupture by ultrasonic oscillation as previously described. The ruptured pellet was centrifuged (105,000 x g for 2 hr at 4°C) and the supernatant was collected by carefully pipetting in order not to disturb the pellet. The resulting cell free extract was used to assay MDH activity as previously described. Results are shown in Table 4.

Mutants 133 and 539 exhibited little or no activity for mannitol

Table 4.

Mannitol Uptake, Mannitol Binding Protein (MBP), and Mannitol Dehydrogenase (MDH) Activities in Spontaneous Revertants of Mannitol Negative Mutant Strains

Strain	Uptake (nmole/min/mg Protein)	MBP (Units/mg)	MDH (Units/mg)
PAO (Wild Type)	34.0	91.4	30.0
105	34.8	131	0.12
105-R	31.2	100	71.2
133	< 1	< 5	0.24
133-R	35.4	84.6	25.6
539	< 1	< 5	< 0.1
539-R	36.3	86.2	25.8
555	< 1	< 5	3.38
555-R	32.8	37.6	31.3
601	< 1	28.6	0.74
601-R	22.3	88.6	32.5

¹Experimental details are given in Materials and Methods section and text of Results section. Activities of Uptake, MBP, and MDH are indicated as <1, <5, and <0.1, respectively, when not detectable and may be zero.

uptake, mannitol binding protein, and mannitol dehydrogenase as compared to the parental PAO phenotype. Revertants of 133 and 539 regained almost complete activity for all three assays. Mutant 555 exhibited no mannitol uptake activity, no mannitol binding protein activity, and low mannitol dehydrogenase activity. The revertant 555-R was able to regain complete uptake activity and mannitol dehydrogenase activity but only half of the parental mannitol binding protein activity. Mutant 601 had no mannitol uptake activity and no mannitol dehydrogenase activity but did exhibit partial MBP activity. The 601 revertant regained partial mannitol uptake activity and complete mannitol binding protein and mannitol dehydrogenase activities. The mutant 105 was able to transport but not catabolize mannitol; mannitol uptake and mannitol binding protein activities were present but mannitol dehydrogenase activity was absent. The 105 revertant regained mannitol dehydrogenase activity with about a 600-fold increase over the 105 mutant and about a two-fold specific activity increase beyond that observed in the parent strain.

Metabolic Inhibition of Mannitol Uptake

The source of energy transduction for the uptake of mannitol was investigated by examining the influence of the metabolic energy inhibitors arsenate and azide. Arsenate is an inorganic phosphate analogue which forms an unstable spontaneously hydrolysing arsenatephosphate ester and thus can be used to uncouple the ATP energy source in metabolically active cells. Azide is a potent inhibitor of the electron transport chain and can be used to impede the normal establishment of

an electrochemical gradient across a cell membrane. Azide can also dissipate an existing proton electrochemical gradient by shuttling hydrogen ions across the membrane.

Cells were grown to mid-exponential phase on 30 mM lactate plus 10 mM mannitol, harvested ($10,000 \times g$ for 10 min at 23°C) washed and resuspended to original culture density with minimal salts medium (37°C), and incubated for one hour (standard conditions). Cells were then harvested and washed twice with TMK buffer, (10 mM Tris-Cl, 1 mM MgCl_2 , 1 mM KCl, pH 7.4) (37°C) and resuspended to a Klett (#66 filter) of 260 (ca. $450 \mu\text{g}$ protein per ml) with TMK buffer, TMK buffer plus 1 mM arsenate, and TMK buffer plus 3 mM azide. Cells were incubated in a large flask in a gyratory shaker (37°C , 250 RPM) for one hour and samples were removed for mannitol uptake assay as previously described. Results are shown in Figure 1. Cells treated with arsenate and azide showed significant inhibition of mannitol uptake as compared to the control cells which were simply incubated in TMK buffer.

Catabolite Repression Resistant Mutant Analysis

A succinate resistant mannitol uptake mutant, designated WEP-1, was selected, screened, and isolated as described in Materials and Methods. Preliminary analysis of the selection methods suggested that WEP-1 was a mannitol transport constitutive mutant.

In order to establish that WEP-1 was a constitutive transport mutant, WEP-1 cells were grown on noninducing and inducing media for mannitol uptake and compared to parent (PAO) cells grown under the

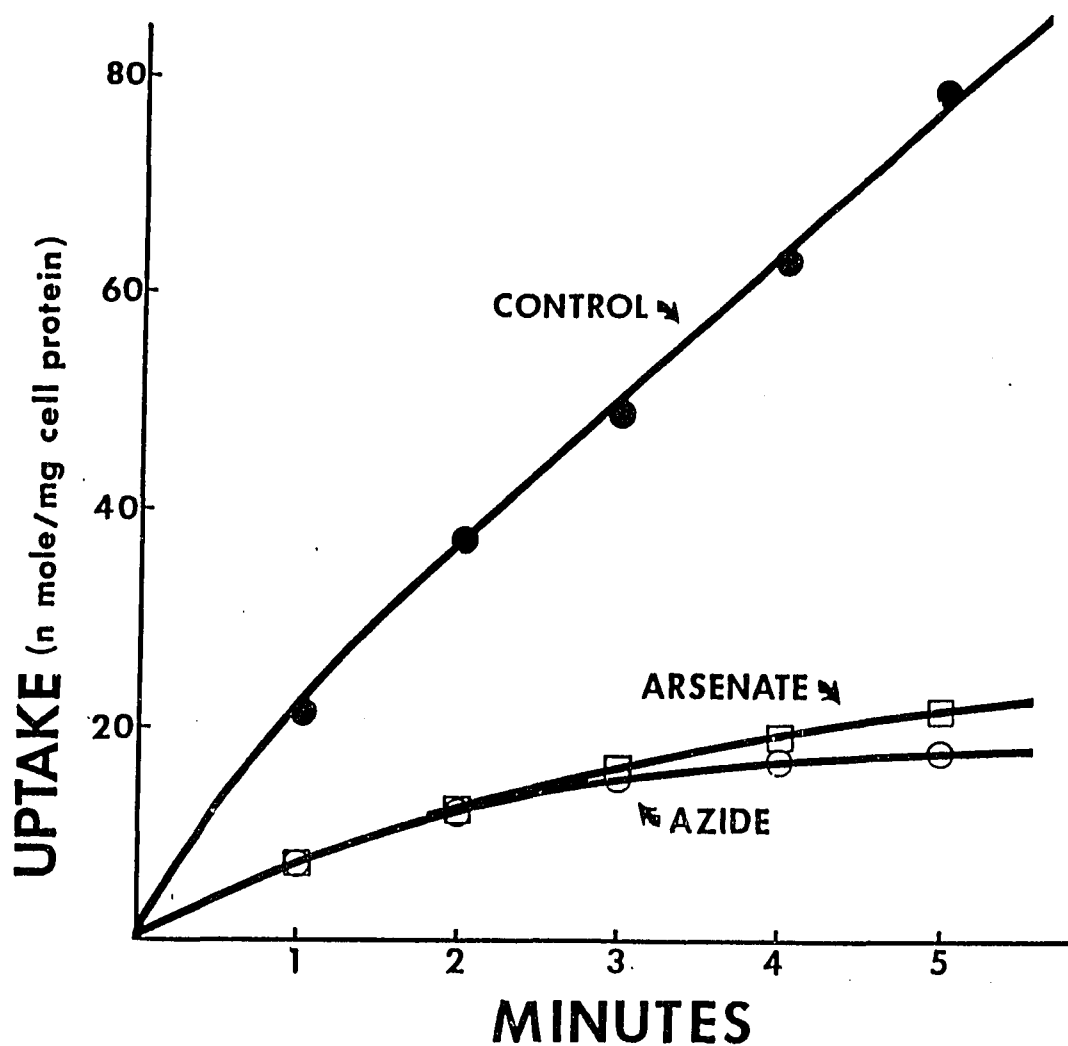


Figure 1. Inhibition of Mannitol Uptake in PAO. (Cells were incubated for one hour in the presence of TMK buffer [●], TMK buffer plus mM arsenate [□], and TMK buffer plus 3 mM azide [○], then assayed for mannitol uptake.)

same conditions. The results of these experiments are given in Table 5 and clearly show that WEP-1 was not a mannitol uptake constitutive mutant since mutant cells grown on 30 mM lactate alone were unable to transport mannitol. Mutant cells, however, were induced to the same extent as parent cells when grown in the presence of lactate plus mannitol; WEP-1 had, therefore, an inducible mannitol uptake system.

There was a significant difference between WEP-1 and PAO for mannitol uptake when mutant and parent cells were grown in the presence of succinate and mannitol (Table 5). Thus, PAO was catabolite repressed for mannitol uptake by succinate as expected, but WEP-1 had a nearly wild-type level of mannitol uptake activity; WEP-1 appeared to be resistant to succinate mediated catabolite repression of normal mannitol induction of mannitol uptake activity.

Table 5.

Mannitol Uptake in PAO Cells and WEP-1 Cells Grown
on Various Carbon and Energy Sources¹

Strain	Carbon and Energy Sources		
	30 mM Lactate	30 mM Lactate 10 mM Mannitol	20 mM Succinate 10 mM Mannitol
PAO	1.0	34	5.7
WEP-1	1.0	40	27

¹Cells were grown on carbon and energy sources indicated, harvested at mid-exponential phase and assayed for ¹⁴C-mannitol uptake activity as described in Materials and Methods. Succinate plus mannitol cells were harvested during mid-exponential phase due to succinate growth (see Figure 2 for differential growth on this combination of energy and carbon sources).

These results (Table 5) and the possibility that WEP-1 is a catabolite repression resistant (crr) mutant suggested that WEP-1 should, if it is a crr phenotype, escape diauxy. Mid-exponential cells, growing on 40 mM succinate, were used to inoculate (5% v/v inoculum) 250 ml prewarmed side arm flasks containing 50 ml minimal salts medium plus limited succinate (6 mM) and either 20 mM glucose or 20 mM mannitol. Results are shown in Figure 2. The PAO control shows a typical diauxy (biphasic) growth pattern for the bacteria preferentially using succinate as a carbon and energy source. The WEP-1 growth pattern was also biphasic indicating the mutant was probably still catabolite repressed in the ability to fully utilize mannitol or glucose in the presence of succinate.

The WEP-1 mutant was assayed for growth and mannitol uptake activity in the presence of both the specific inducer, mannitol and the strong catabolite repressor, succinate. Cells grown under standard conditions to mid-exponential phase on 40 mM succinate as sole carbon and energy source were harvested, washed with buffer A (37°C) and used to inoculate Fernback flasks containing 1000 ml prewarmed and aerated minimal salts medium, plus 20 mM mannitol and 10 mM succinate. Cultures were inoculated to give a Klett of 40 and assayed for both growth and mannitol uptake as a function of time. Results presented in Figure 3 show that WEP-1 and the parent PAO both grow at approximately the same exponential rates. However, WEP-1 exhibits a shorter lag period before entering the exponential growth phase. A more significant difference between WEP-1 and PAO is evident when comparing the uptake of mannitol. As seen in Figure 3, both cultures

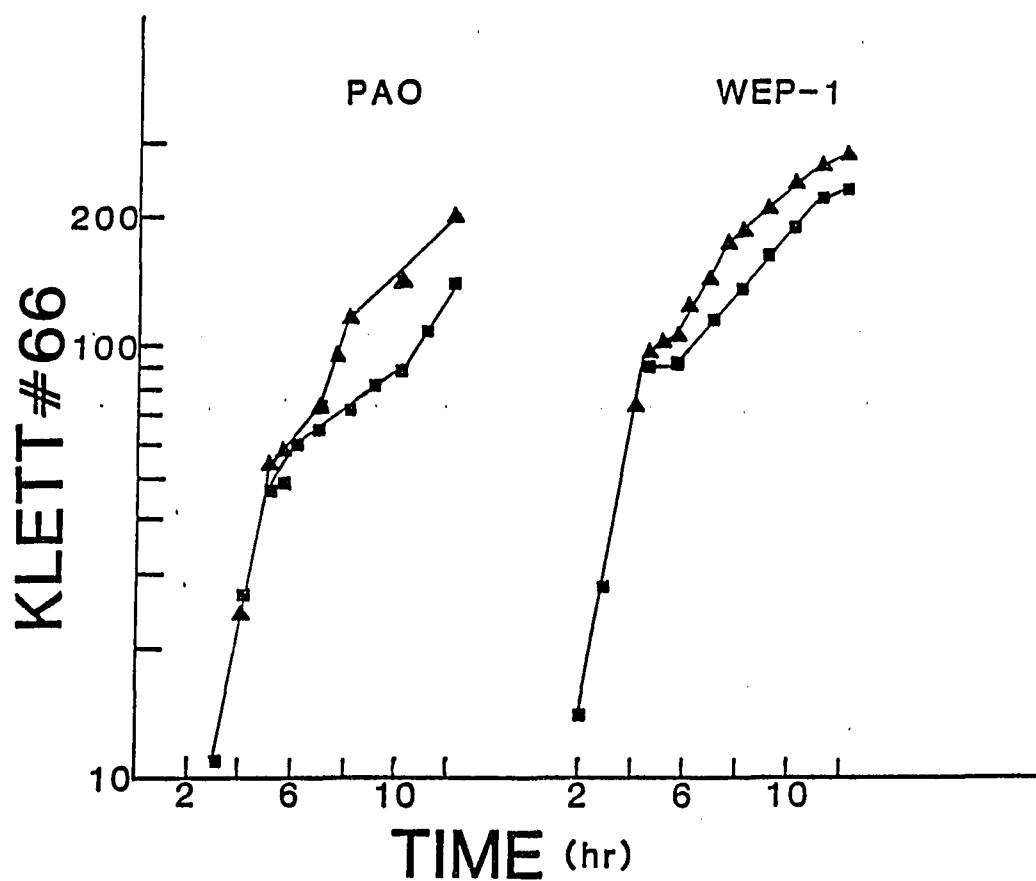


Figure 2. Diauxic Growth of PAO and WEP-1. (Growth of PAO and WEP-1 cultures on 6 mM succinate plus 20 mM glucose [▲], or 20 mM mannitol [■]. Side arm flasks containing 50 ml minimal salts medium plus indicated carbon and energy sources were inoculated with 2.5 ml of mid-exponentially growing culture in 20 mM succinate and were monitored for growth with a Klett #66 filter.)

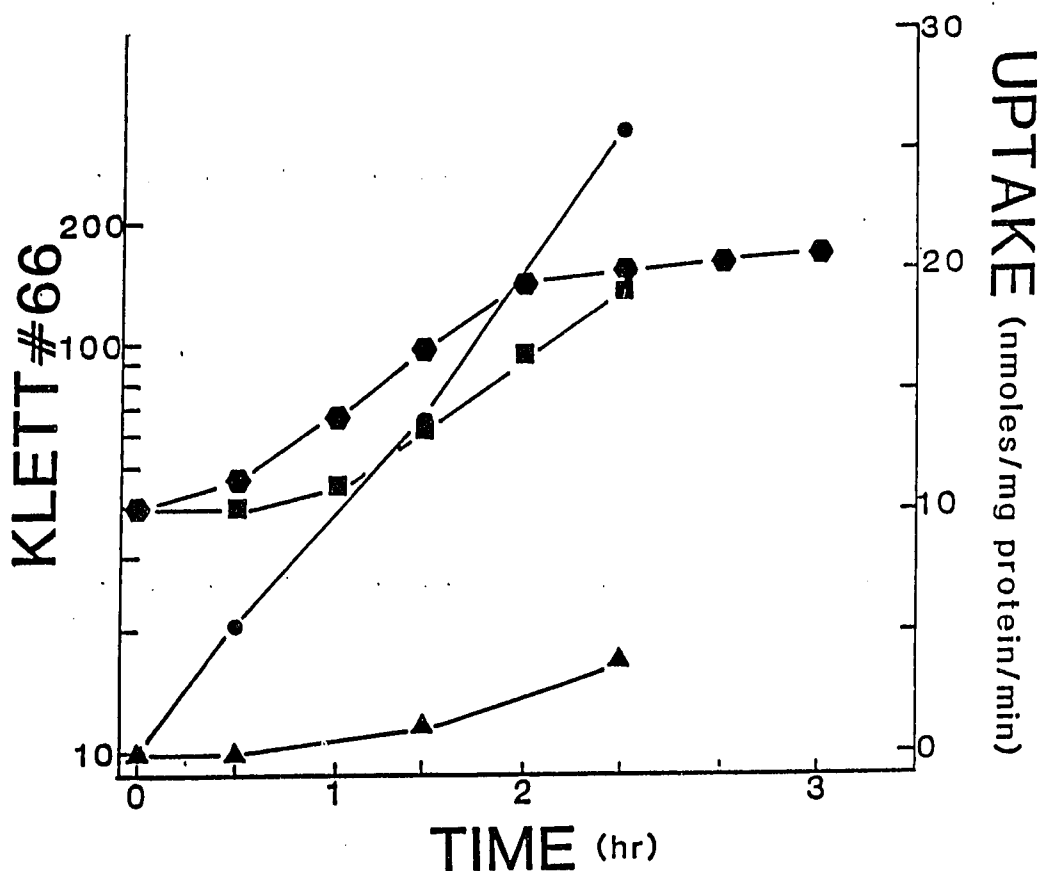


Figure 3. Repressed Mannitol Uptake Induction in PAO and WEP-1. (Uptake of mannitol in cell cultures growing in minimal salts medium with 10 mM succinate plus 20 mM mannitol. Fernback flasks were inoculated at time zero with washed cells harvested from mid-exponential growth in 20 mM succinate. Growth of PAO [■] and WEP-1 [●] were monitored with a Klett #66 filter. Mannitol uptake in PAO [▲] and WEP-1 [●] was determined during corresponding culture growth.)

initially exhibit low mannitol uptake activities. However, at 2.5 hours (approximately two generations) after the addition of mannitol (inducer) the WEP-1 culture has a substantially greater degree of mannitol uptake activity than the PAO culture. The significance of this difference in mannitol uptake activity is better defined by the differential plot as depicted in Figure 4. In the differential plot the specific activity of mannitol uptake is unequivocally compared between different cultures as a function of culture density (Klett) rather than time. From Figure 4, the rate of mannitol uptake activity increases to approximately six times greater in the mutant as compared to the wild-type after the addition of mannitol inducer.

The catabolite repression by succinate of the mannitol specific peripheral pathway enzymes (mannitol dehydrogenase and fructokinase), as well as glucose-6-phosphate dehydrogenase, and the Entner-Doudoroff pathway enzymes (6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase) was examined in PAO and the WEP-1 mutant. Early exponential phase cultures growing under standard conditions on 40 mM mannitol were harvested ($10,000 \times g$ for 10 min at 23°C), resuspended with minimal salts media and used to inoculate fresh minimal salts media containing 40 mM mannitol to a Klett of approximately 10 to 20. Freshly inoculated culture growing in exponential phase under standard conditions were supplemented with 40 mM sodium succinate (pH 7.0) at a Klett of 60 (ca. mid-exponential growth). Culture samples (up to 400 ml) were harvested at various times up to a Klett of 200. Samples were collected by centrifugation ($10,000 \times g$ for 10 min at 23°C) washed once with buffer A (23°C), recentrifuged and

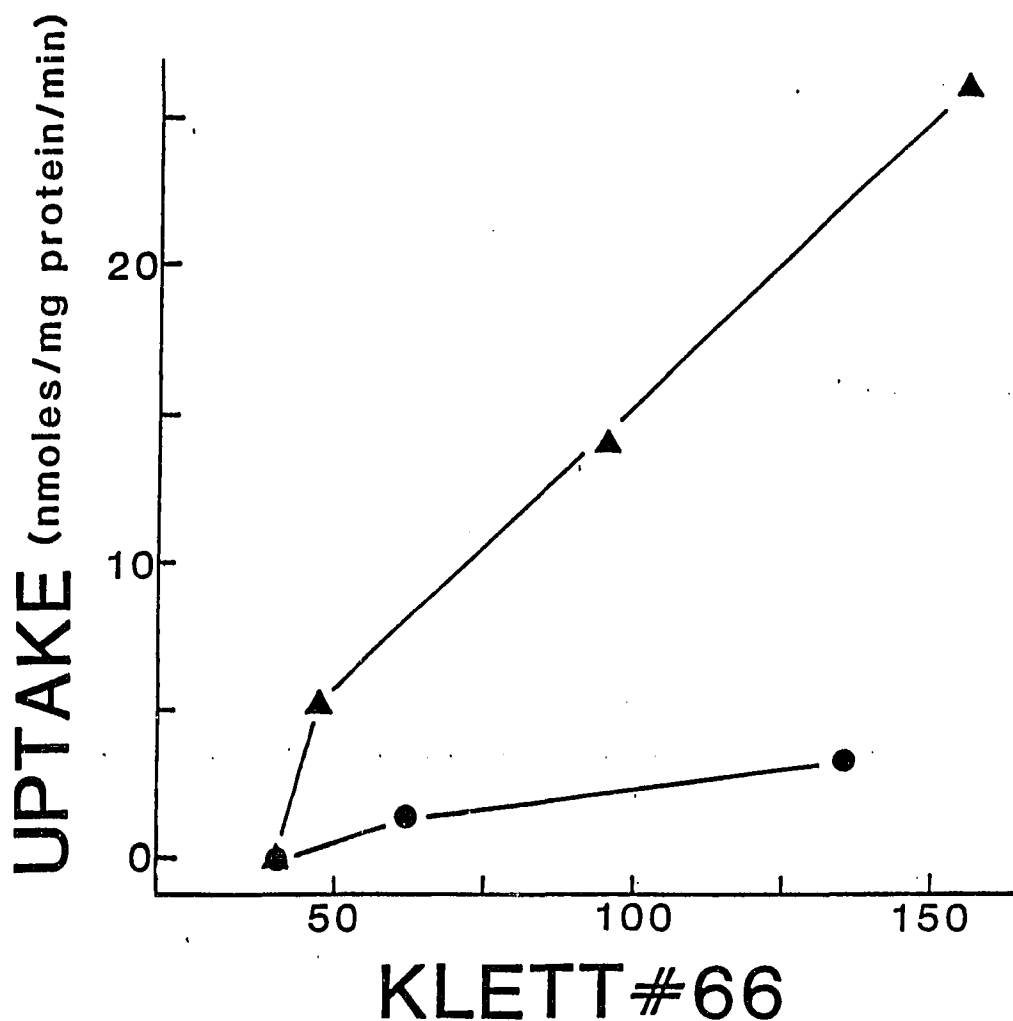


Figure 4. Differential Plot of Repressed Mannitol Uptake Induction in PAO and WEP-1. (Mannitol uptake induction by PAO [●] and WEP-1 [▲] cultures grown in minimal salts medium containing 20 mM mannitol plus 10 mM succinate. Fernback flasks were inoculated to a Klett of 40 with washed cells harvested from mid-exponential growth in 20 mM succinate.)

frozen (-70°C) overnight. Frozen samples were thawed and diluted to approximately 5 mg protein per ml with 50 mM Tris-Cl (pH 8.0 at 0°C) plus 10 mM 2-mercaptoethanol. Samples were ruptured by ultrasonic oscillation, centrifuged ($105,000 \times g$ for 2 hr at 4°C) and the resulting supernatant, collected by pipette, was assayed as previously described. Results are presented graphically as a growth curve plus enzyme activities for PAO in Figure 5 and WEP-1 in Figure 6. Results from these same experiments are also presented as differential plots, Figure 7, showing enzyme activities as a function of culture density (Klett). Enzyme activity for a given sample is presented as a percentage of the activity in that culture sample which displayed the greatest specific activity. The results depicted in Figures 5, 6, and 7 show that specific enzyme activities are strongly repressed by the addition of succinate to WEP-1 and PAO cultures growing on mannitol.

Figure 5. Catabolite Repression of Mannitol Induced Enzymes in PAO. (Cells growing on 40 mM mannitol were supplemented with 40 mM succinate at a level of growth indicated by a Klett of approximately 60 [⊙]. Culture samples were removed at time periods just before and after the addition of succinate and enzyme specific activities were determined. Enzyme activities are represented as a percentage of the maximal specific activity for a given enzyme. Enzyme, maximal specific activity (mI.U. per mg protein), and graph symbols are indicated below: mannitol dehydrogenase, 56, [●]; fructokinase, 60, [▲]; glucose-6-phosphate dehydrogenase, 402, [■]; 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase combined activities, 117, [⊙].)

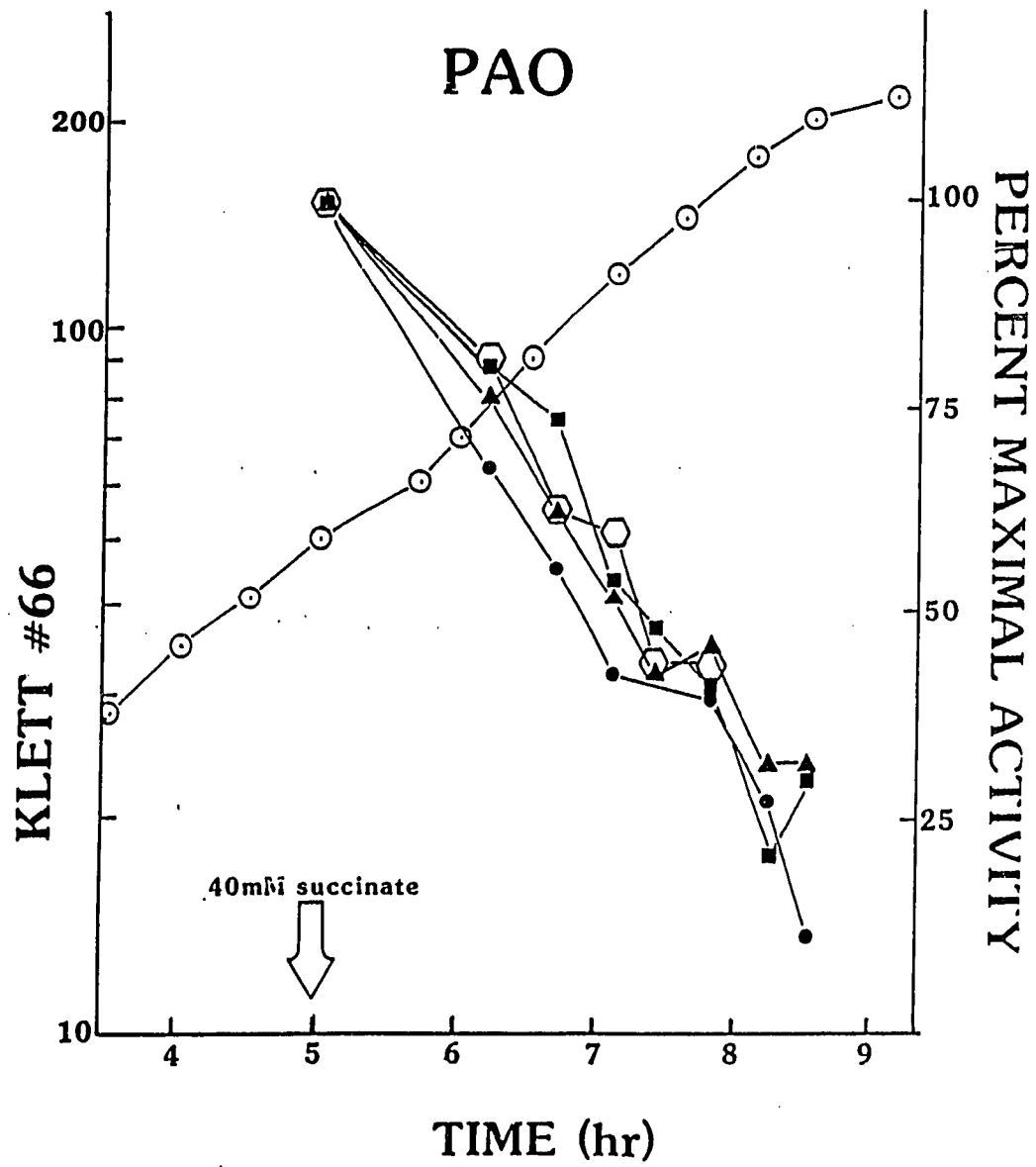


Figure 5. Catabolite Repression of Mannitol Induced Enzymes in PAO.

Figure 6. Catabolite Repression of Mannitol Induced Enzymes in WEP-1. (Cells growing on 40 mM mannitol were supplemented with 40 mM succinate at a level of growth indicated by a Klett of approximately 60 [⊙]. Culture samples were removed at time periods just before and after the addition of succinate and enzyme specific activities were determined. Enzyme activities are represented as a percentage of the maximal specific activity for a given enzyme. Enzyme, maximal specific activity (mI.U. per mg protein), and graph symbols are indicated below: mannitol dehydrogenase, 46, [⊙]; fructokinase, 65, [▲]; glucose-6-phosphate dehydrogenase, 360, [■]; 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase combined activities, 116, [⊙].)

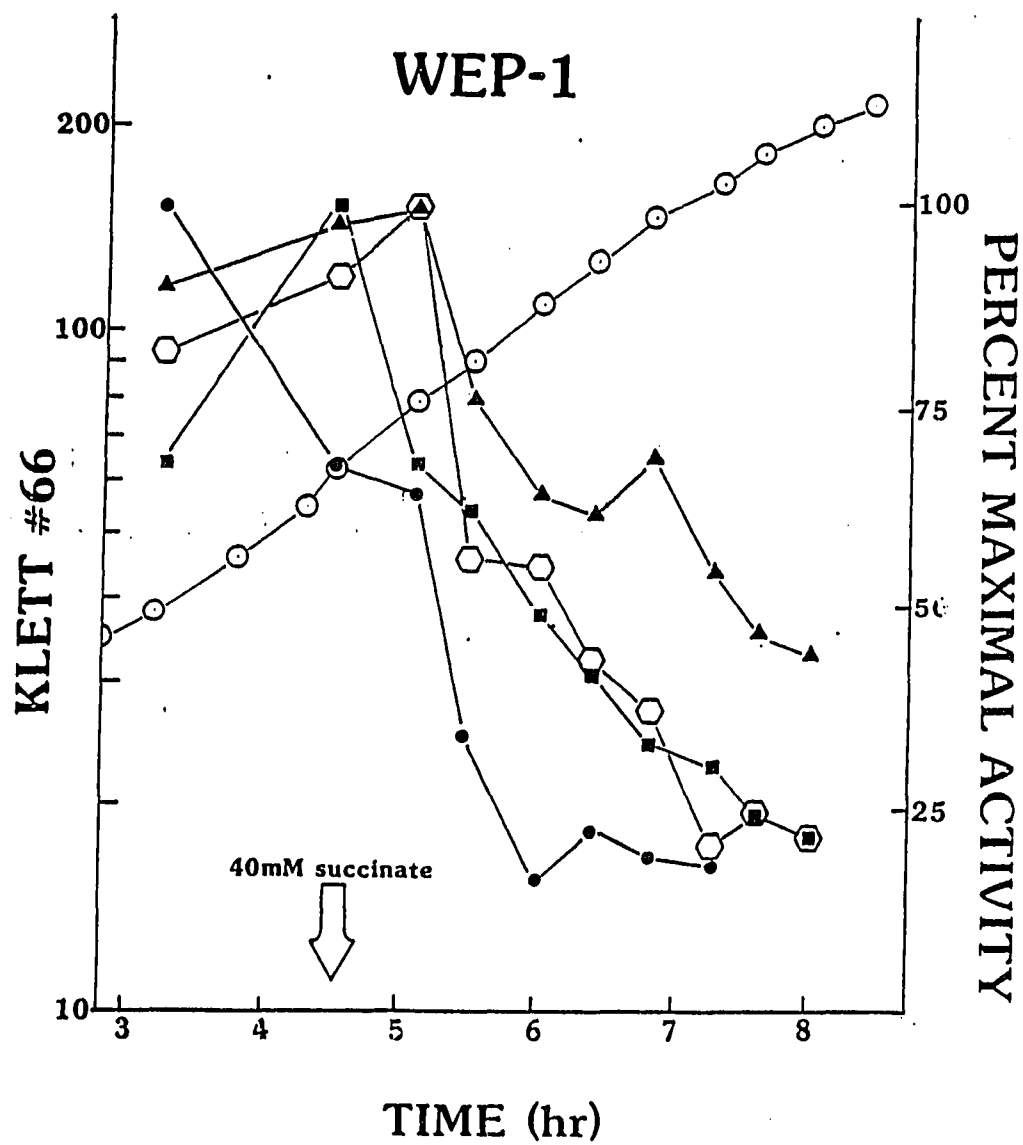


Figure 6. Catabolite Repression of Mannitol Induced Enzymes in WEP-1.

Figure 7. Differential Plots of Catabolite Repression of Mannitol Induced Enzymes in PAO and WEP-1. (Cells growing on 40 mM mannitol were supplemented with 40 mM succinate at a level of growth indicated by a Klett of approximately 60. Culture samples, at various times after inoculation, were assayed for mannitol dehydrogenase [●], fructokinase [▲], and glucose-6-phosphate dehydrogenase [■] activities as well as the combined activities of 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase [⊕]. Enzyme activity is presented as a percentage of the maximal specific activity for a given enzyme. Specific values are listed in the legends of Figures 5 and 6.)

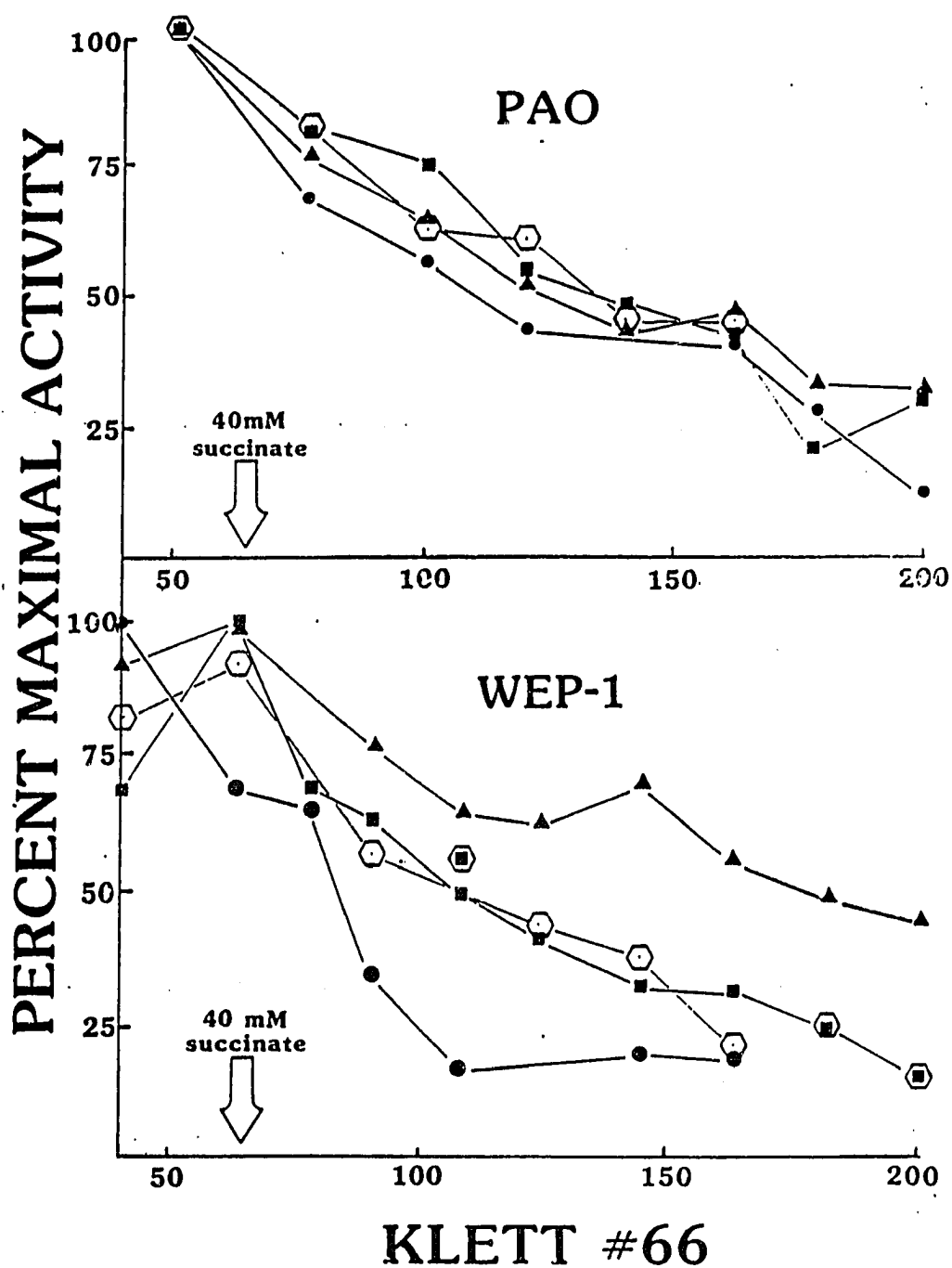


Figure 7. Differential Plots of Catabolite Repression of Mannitol Induced Enzymes in PAO and WEP-1.

DISCUSSION

Two basically different cold-shock procedures were used to determine the intracellular distribution of mannitol binding protein. In both procedures glucose-6-phosphate dehydrogenase (a cytoplasmic enzyme) was employed as a control marker to demonstrate leakage of the cytoplasm or cell lysis. In the lysozyme cold-shock experiment, 25% of the total glucose-6-phosphate dehydrogenase activity was located in the spheroplast supernatant fraction; the remaining 75% being found in the cytoplasmic fraction (see Table 1). This indicates extensive cytoplasmic leakage probably due to cell lysis and which might be expected considering the fragility of spheroplast membranes. At the same time, the opposite distribution occurred for mannitol binding protein activity; 75% of the total MBP activity was located in the spheroplast supernatant fraction and 25% in the cytoplasmic fraction. If one assumes that mannitol binding protein is to be found exclusively in the periplasm (as is true of bacterial binding proteins generally), one can rationalize this skewed distribution by assuming that not all cells were transformed into spheroplasts or that some cells were only partially transformed. Thus, periplasmic components may have contaminated the cytoplasmic fraction when the shock pellet was lysed.

Another possibility is that the shock pellet was not washed of significant periplasmic contamination before being lysed. The membrane fraction was devoid of any significant activity for either protein. Strictly on the basis of this data, taking into account the

known cross contamination of cytoplasmic marker protein into the spheroplast fraction (and presumed reverse contamination of the cytoplasmic fraction), mannitol binding protein appears to be located primarily in the periplasmic region of the cell. Fortunately, the results from the magnesium chloride cold-shock procedure were able to clarify some of this ambiguity.

In the magnesium chloride cold-shock experiment, virtually all of the glucose-6-phosphate dehydrogenase activity remained in the shock pellet fraction, thus indicating no leakage (lysis) of cell cytoplasm into the shock supernatant (see Table 2). The mannitol binding protein activity was concentrated almost completely in the shock supernatant fraction, 92% of the total, with only 8% recovered in the shock pellet. Again, it may be assumed that some of the cells remained refractile to the shock treatment and when shocked cells were lysed, the refractile cells were then able to contribute periplasmic contaminants to the shock pellet which was used to prepare the cytoplasmic fraction. Washing of the shock pellet before lysis may have been able to reduce some of this contamination. Clearly, the distribution of binding protein activity (Table 2) presents excellent evidence that mannitol binding protein is a periplasmic protein.

The use of magnesium chloride in the cold-shock buffer was necessary for efficient mannitol binding protein extraction. When magnesium chloride was not included in the shock buffer, much less protein was included (see Table 3). The proteins that were released by the simple cold-shock treatment were almost four times more spe-

cific for mannitol binding protein activity than the proteins released in the magnesium chloride treatment. Cold-shock cells showed a ten-fold reduction in mannitol uptake activity compared to control cells which were not cold-shocked. Loss of binding protein with concomitant loss of transport or uptake activity has been documented for other known shock sensitive binding protein transport systems (Anraku, Kobayashi, Amanuma & Yamaguchi, 1973; Oxender & Quay, 1976; Wilson & Holden, 1969).

This relationship between the loss of transport and the loss of binding protein activity is better defined by the analysis of mutants which are deficient in these functions. The results of such an analysis (see Table 4) strongly implicates mannitol binding protein as an obligatory component for the transport of mannitol. It is interesting to note that all mutants had reversion frequencies at approximately 0.2×10^{-9} which, being a relatively high frequency of repair, suggests that these are single site lesions (point mutations) on the chromosome. Mutants 133, 539, and 555 were found deficient in both mannitol binding protein and mannitol dehydrogenase activity and revertants regained activity for both functions. If one assumes that these are point mutations, this apparent pleiotropic phenotype might be caused by a nonsense mutation exerting a polar effect on a gene distal to the lesion but located within a common operon. Alternatively, the mutation might be "trans" and acting as a diffusible regulatory element that affects a common regulon. Another possibility could be that mannitol transport mutants prevent induction of mannitol dehydrogenase because the inducer (mannitol) is excluded from the

interior of the cell. Other theories may also be plausible. However, they should be able to explain the data concerning mutant 601. In the case of mutant 601, mannitol uptake is abolished, mannitol binding protein activity is reduced to a third of the wild-type value and there is no mannitol dehydrogenase activity. Again, the spontaneous revertant regains all these activities at levels comparable to wild-type. This is an unusual phenotype that exhibits only reduced binding protein activity and yet completely fails to transport mannitol. This could be due to a mutation in the MBP gene that results in making a gene product which is defective in interaction with a membrane protein. However one chooses to interpret these data, the overall evidence for all mutants and revertants argues strongly that mannitol binding protein is, in fact, an obligatory component for the transport of mannitol.

The metabolic inhibition experiment corroborates the report that mannitol is acquired by an active transport mechanism (Eagon & Phibbs, 1971) and also suggests that energization of mannitol transport is mediated by ATP rather than an electrochemical gradient. From Figure 1, it can be seen that when cells are treated with low concentrations of either arsenate or azide the result is potent inhibition of mannitol uptake activity as compared to the control experiment where cells were untreated by inhibitors.

If energization of mannitol transport occurred by direct symport/antiport via an electrochemical gradient, one would expect arsenate to have little effect on transport activity (arsenate uncouples synthesis of ATP; the electrochemical gradient remains unaffected).

Azide, however, should cause extensive inhibition of uptake activity regardless of the required energy source because, in the case of Pseudomonas and other obligate aerobes, ultimately almost all ATP is derived from oxidative phosphorylation. With respect to mannitol transport, arsenate effectively reduced uptake activity to a level almost identical to that caused by azide inhibition. Thus, it would seem plausible that mannitol transport requires ATP as an energy donor and, this too, is commensurate with what is known about energy requirements in other binding protein active transport systems (Berger, 1973; Berger & Heppel, 1974; Ferenci et al., 1977; Romano et al., 1980).

As a further effort to obtain supporting evidence for the role of the inducible mannitol binding protein as an obligatory component in the transport of mannitol, a search was initiated for the selection of a constitutively expressed mannitol binding protein mutant. In addition to the intrinsic value as corroborating evidence, such a constitutive mutant could prove useful as starting material for the large scale isolation and purification of mannitol binding protein. With this intention, the selection procedure described in the Materials and Methods section, which ultimately yielded the WEP-1 mutant, was initiated. The basic procedure consists of transferring a mannitol induced culture to a non-inducing medium which prevents the expression of the mannitol induced proteins. After sufficient growth, the mannitol repressed culture is transferred from the non-inducing medium back to the mannitol minimal medium in order to begin induction of mannitol specific proteins so that growth may resume

utilizing mannitol as the sole carbon and energy source. In theory, the enrichment for a mannitol constitutive strain should occur when cultures are transferred from the non-inducing medium to the inducing medium. Normally, during this time, cultures exhibit a considerable lag in growth. This occurs because the single carbon and energy source available for growth, mannitol, must remain inaccessible until such time has passed to allow for the sufficient induction of mannitol specific catabolic proteins. A mutation which allows constitutive expression of the mannitol specific proteins could prevent this transient carbon and energy crisis during transfer from the non-inducing medium to mannitol minimal medium. This would allow the mutant to initiate growth on mannitol more quickly than wild-type cells. Such a mutant would multiply by a few generations while the wild-type cells were unable to grow. If selective pressure is maintained for a sufficient period of time by cyclic transfer of the culture between the non-inducing and inducing media, a mutant population as described above would be expected to eventually succeed the slower growing wild-type cells. By this method, a putative mannitol constitutive mutant, designated WEP-1, was obtained.

The choice of succinate as a non-inducing carbon source was important because this substrate enhances conditions of selection for a constitutive mutant. Succinate, in addition to not inducing the mannitol specific proteins, also causes strong catabolite repression of the inducible pathways of carbohydrate catabolism including the mannitol induced proteins as well as the Entner-Doudoroff enzymes and glucose-6-phosphate dehydrogenase. The screening protocol required

that the constitutive mutant be able to demonstrate mannitol utilization under conditions for which such activity would normally not be present. Screening a culture for the presence of a mannitol uptake constitutive mutant basically consisted of spreading cells onto a solid medium containing succinate and radiolabeled mannitol. The resulting colonies which exhibited incorporation of label (as demonstrated by autoradiography) would be either, resistant to succinate driven catabolite repression for the induction of mannitol transport and possibly downstream inducible enzymes, or mutants which constitutively express mannitol uptake.

The first experiment was designed to examine if WEP-1 was a constitutive uptake mutant by determining the activity of mannitol transport (uptake) under growth conditions containing inducer (mannitol), non-inducer (lactate), and inducer plus repressor (mannitol plus succinate). The results from these experiments are summarized in Table 5. Under non-inducing growth conditions for mannitol utilization, no detectable mannitol uptake activity was present in either the wild-type control or the WEP-1 mutant. Clearly, the WEP-1 mutant was not constitutively expressing mannitol transport activity. When cells were grown in the presence of inducer, mannitol uptake was expressed at slightly higher levels in the WEP-1 mutant as compared to the induced PAO control. However, under repressive conditions in the presence of inducer (succinate plus mannitol) the WEP-1 mutant exhibited a five-fold increase in uptake activity over wild-type levels. Thus, the phenotype detected in the mutant screening procedure was not the result of a constitutive mannitol transport mutation but rather the re-

sult of an ability of the mutant to induce mannitol uptake activity significantly beyond normal levels under conditions which cause strong catabolite repression in normal cells.

After establishing that WEP-1 was not a constitutive mutant, diauxic growth experiments were performed to demonstrate the ability for the mutant to continue growing when switching from the repressive carbon source, succinate, to the inducible carbon source, mannitol. In addition to mannitol, several other inducible carbon sources were used as control experiments to demonstrate the specificity of the mutation. The results of some of these diauxic growth experiments are shown in Figure 1. From these data it was clear that no significant differences could be discerned between the biphasic growth curves of the various inducible carbon sources or between the putative constitutive mutant, WEP-1, and the PAO wild-type. These results suggested that the assumed catabolite repression resistant (crr) phenotype was incorrect or only partially correct. In the case of mannitol catabolism, at least three inducible mannitol specific proteins must be present before the carbohydrate can be utilized. They include the mannitol binding protein for transport, mannitol dehydrogenase, and fructokinase activities. In addition, glucose-6-phosphate dehydrogenase and Entner-Doudoroff enzymes must be induced before significant mannitol catabolism can occur. Perhaps inducible proteins early in the mannitol pathway were crr expressed in WEP-1 but the downstream enzymes remained inducible and under strong catabolite repression control.

The next experiment was designed to examine the kinetics of man-

nitrol uptake induction under repressive growth conditions in the WEP-1 mutant and the PAO control. Cultures of PAO and WEP-1 which were preadapted for growth on succinate were transferred to a medium containing succinate plus mannitol. Both cultures grew at approximately equal rates (compare the slopes of exponential growth, Figure 3). The induction of mannitol uptake activity in the WEP-1 was faster than in PAO (see Figure 2) and WEP-1 reached steady state levels of activity five times greater than PAO (see Table 5). This apparent mannitol transport escape of catabolite repression was unable to prevent the biphasic growth curve evident under diauxic growth conditions (see Figure 1).

In order to better understand the biphasic characteristics of diauxic growth in WEP-1, a repression experiment was designed to analyze the extent to which downstream mannitol catabolic pathway enzymes remained under catabolite repressing control. When WEP-1 and PAO cultures induced for steady-state growth on mannitol are supplemented with succinate, all inducible downstream enzymes are immediately shut down (see Figures 5, 6, and 7). With respect to PAO, the trend appears evenly downward for all enzymes. In WEP-1, the trend is also downward for all enzymes but shut-down rates appear to vary. How this may affect physiological growth characteristics is not clear and, in fact, it remains to be determined whether the differences seen in the rates of shut-down are physiologically significant or whether they influence metabolic changes apart from the obvious cessation of mannitol catabolism.

The mechanism of catabolite repression in P. aeruginosa is unknown.

Attempts to define a c-AMP mediated mechanism for catabolite repression analogous to the system found in Enterobacteriaceae have met with little success (Phillips & Mulfinger, 1981; Shapiro et al., 1972; Siegel et al., 1977). These attempts have not invalidated the idea that a common control principle may be at work in Pseudomonas much in the same way that the c-AMP binding protein has its effect over a broad spectrum of otherwise independent operons, i.e., as a c-AMP mediated regulon (Pastin & Adhya, 1973). If catabolite repression in PAO also operates to control a regulon of repressible activities, then perhaps the resistance to catabolite repression that has occurred in WEP-1 is confined to mannitol transport by a mannitol promoter-operator mutational event, i.e., a "cis" mutation. This would explain why downstream enzymes appear to remain under catabolite repression control.

The question of selective advantage still remains. Physiologically, what allowed WEP-1 to grow to dominate the selection-enrichment culture? Perhaps mannitol uptake is able to induce more quickly in WEP-1 than PAO because induction is now independent of catabolite repression. As soon as inducer is present the mannitol operon could be derepressed because expression would no longer have to wait for the metabolic decay of a negative control element or for the build-up of a positive control element. Also, cells which are rapidly loaded with mannitol could be derepressed in other downstream enzymes by virtue of a high concentration of inducer. A closer examination of growth rate during diauxic lag may reveal a slight growth advantage in the WEP-1 which is not evident under less refined experimental conditions

for measuring growth as were used in this study.

BIBLIOGRAPHY

- Adler, J. Chemoreceptors in Bacteria: Studies of Chemotaxis Reveal Systems that Detect Attractants Independently of Their Metabolism. Science, 1969, 166, 1588-1597.
- Adler, J. Chemotaxis in Bacteria. Annual Review of Biochemistry, 1975, 44, 341-376.
- Aksamit, R., & Koshland, D. E., Jr. A Ribose Binding Protein of Salmonella typhicurium. Biochemical and Biophysical Research Communications, 1972, 48, 1348-1353.
- Ames, G. F. -L., & Lever, J. Components of Histidine Transport: Histidine-Binding Proteins and hisP Protein. Proceedings of the National Academy of Sciences, 1970, 66, 1096-1103.
- Ames, G. F. -L., & Nikaido, K. Identification of a Membrane Protein as a Histidine Transport Component in Salmonella typhimurium. Proceedings of the National Academy of Sciences, 1978, 75, 5447-5451.
- Ames, G. F. -L., & Spudich, E. N. Protein-Protein Interaction in Transport: Periplasmic Histidine-Binding Protein J Interacts with P Protein. Proceedings of the National Academy of Sciences, 1976, 73, 1877-1881.
- Anraku, Y. Transport of Sugars and Amino Acids in Bacteria, I., Purification and Specificity of the Galactose- and Leucine-Binding Proteins. Journal of Biological Chemistry, 1968, 243, 3116-3122. (a)
- Anraku, Y. Transport of Sugars and Amino Acids in Bacteria, II., Properties of Galactose- and Leucine-Binding Proteins. Journal of Biological Chemistry, 1968, 243, 3123-3127. (b)
- Anraku, Y., Kobayashi, H., Amanuma, H., & Yamaguchi, A. Transport of Sugars and Amino Acids in Bacteria., VII. Characterization of the Reaction of Restoration of Active Transport Mediated by Binding Protein. Journal of Biochemistry, 1973, 74, 1249-1261.
- Berger, E. A. Different Mechanisms of Energy Coupling for the Active Transport of Proline and Glutamine in Escherichia coli. Proceedings of the National Academy of Sciences, 1973, 70, 1514-1518.
- Berger, E. A., & Heppel, L. A. A Binding Protein Involved in the Transport of Cystine and Diaminopimelic Acid in Escherichia coli. Journal of Biological Chemistry, 1972, 247, 7684-7694.

- Berger, E. A., & Heppel, L. A. Different Mechanisms of Energy Coupling for the Shock-Sensitive and Shock-Resistant Amino Acid Permeases of Escherichia coli. Journal of Biological Chemistry, 1974, 249, 7747-7755.
- Boos, W. Bacterial Transport. Annual Review of Biochemistry, 1974, 43, 123-146.
- Cheng, K. -J., Ingram, J. M., & Costerton, J. W. Release of Alkaline Phosphatase from Cells of Pseudomonas aeruginosa by Manipulation of Cation Concentration and pH. Journal of Bacteriology, 1970, 104, 748-753. (a)
- Cheng, K. -J., Ingram, J. M., & Costerton, J. W. Alkaline Phosphatase Localization and Spheroplast Formation of Pseudomonas aeruginosa. Canadian Journal of Microbiology, 1970, 16, 1319-1324. (b)
- Eagon, R. G., & Carson, K. J. Lysis of Cell Walls and Intact Cells of Pseudomonas aeruginosa by Ethylenediamine Tetraacetic Acid and by Lysozyme. Canadian Journal of Microbiology, 1965, 11, 193-201.
- Eagon, R. G., & Phibbs, P. V., Jr. Kinetics of Transport of Glucose, Fructose, and Mannitol by Pseudomonas aeruginosa. Canadian Journal of Biochemistry, 1971, 49, 1031-1041.
- Eisenberg, R. C., & Phibbs, P. V., Jr. Detection of an Inducible Mannitol-Binding Protein in Pseudomonas aeruginosa. Virginia Journal of Science, 1978, 29 (2): 104.
- Eisenberg, R. C., & Phibbs, P. V., Jr. Mannitol Binding Protein in Pseudomonas aeruginosa. Abstracts of the Annual Meeting of the American Society for Microbiology, 1979, 162 (K101).
- Eisenberg, R. C., & Phibbs, P. V., Jr. Characterization of an Inducible Mannitol-Binding Protein from Pseudomonas aeruginosa. Current Microbiology, 1982, 7, 229-234.
- Ferenci, T., Boos, W., Schwartz, M., & Szmecman, S. Energy-Coupling of the Transport System of Escherichia coli Dependent on Maltose-Binding Protein. European Journal of Biochemistry, 1977, 75, 187-193.
- Furlong, C. E. Purification of a Leucine-Specific Binding Protein and Evidence for a Second Leucine Transport System. Federation Proceedings, 1970, 29, 544 (341)
- Gordon, A. S., Lombardi, F. J., & Kaback, H. R. Solubilization and Partial Purification of Amino Acid-Specific Components of the D-Lactate Dehydrogenase-Coupled Amino Acid-Transport Systems. Proceedings of the National Academy of Sciences, 1972, 69, 358-362.

- Hoshino, T., & Kageyama, M. Sodium-Dependent Transport of L-Lucine in Membrane Vesicles Prepared from Pseudomonas aeruginosa. Journal of Bacteriology, 1979, 137, 73-81.
- Hoshino, T., & Kageyama, M. Purification and Properties of a Binding Protein for Branched-Chain Amino Acids in Pseudomonas aeruginosa. Journal of Bacteriology, 1980, 141, 1055-1063.
- Hylemon, P. B., & Phibbs, P. V., Jr. Independent Regulation of Hexose Catabolizing Enzymes and Glucose Transport Activity in Pseudomonas aeruginosa. Biochemical and Biophysical Research Communications, 1972, 48, 1041-1048.
- Kaback, H. R. Transport Across Isolated Bacterial Cytoplasmic Membranes. Biochimica et Biophysica Acta, 1972, 265, 367-416.
- Kalb, V. F., Jr., & Bernlohr, R. W. A New Spectrophotometric Assay for Protein in Cell Extracts. Analytical Biochemistry, 1977, 82, 362-371.
- Kellermann, O., & Szmecman, S. Active Transport of Maltose in Escherichia coli K12. Involvement of a "Periplasmic" Maltose Binding Protein. European Journal of Biochemistry, 1974, 47, 139-149.
- Klein, W. L., & Boyer, P. D. Energization of Active Transport by Escherichia coli. Journal of Biological Chemistry, 1972, 247, 7257-7265.
- Lever, J. E. Purification and Properties of a Component of Histidine Transport in Salmonella typhimurium. The Histidine-Binding Protein J. Journal of Biological Chemistry, 1972, 247, 4317-4326.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. Protein Measurement with the Folin Phenol Reagent. Journal of Biological Chemistry, 1951, 193, 265-275.
- Magasanik, B. Catabolite-Repression. Cold Springs Harbor Symposium on Quantitative Biology, 1961, 26, 249-256.
- Medveczky, N., & Rosenberg, H. The Binding and Release of Phosphate by a Protein Isolated from Escherichia coli. Biochimica et Biophysica Acta, 1969, 192, 369-371.
- Monod, J. The Phenomenon of Enzymatic Adaptation and Its Bearings on Problems of Genetics and Cellular Differentiation. Growth, 1947, 11, 223-289.
- Neu, H. C., & Heppel, L. A. The Release of Enzymes from Escherichia coli by Osmotic Shock and During the Formation of Spheroplasts. Journal of Biological Chemistry, 1965, 240, 3685-3692.

- Nishimune, T., & Hayashi, R. Thiamine-Binding Protein and Thiamine Uptake by Escherichia coli. Biochimica et Biophysica Acta, 1971, 244, 573-583.
- Oxender, D. L., & Quay, S. C. Isolation and Characterization of Membrane Binding Proteins. In E. D. Korn (Ed.), Methods in Membrane Biology (Vol. 6). New York, London: Plenum Press, 1976, pp. 183-242.
- Pardee, A. B., Prestidge, L. S., Whipple, M. B., & Dreyfuss, J. A Binding Site for Sulfate and Its Relation of Sulfate Transport into Salmonella typhimurium. Journal of Biological Chemistry, 1966, 241, 3962-3969.
- Parsons, R. G., & Hogg, R. W. Crystallization and Characterization of the L-Arabinose-Binding Protein of Escherichia coli B/r. Journal of Biological Chemistry, 1974, 249, 3602-3607.
- Pastan, I., & Adhya, S. Cyclic Adenosine 5'-Monophosphate in Escherichia coli. Bacteriological Reviews, 1973, 40, 527-551.
- Pastan, I., & Perlman, R. Cyclic Adenosine Monophosphate in Bacteria. Science, 1970, 169, 339-344.
- Phibbs, P. V., Jr., & Eagon, R. G. Transport and Phosphorylation of Glucose, Fructose, and Mannitol by Pseudomonas aeruginosa. Archives of Biochemistry and Biophysics, 1970, 138, 470-482.
- Phibbs, P. V., Jr., McCowen, S. M., Feary, T. W., & Blevins, W. T. Mannitol and Fructose Catabolic Pathways of Pseudomonas aeruginosa Carbohydrate-Negative Mutants and Pleiotropic Effects of Certain Enzyme Deficiencies. Journal of Bacteriology, 1978, 133, 717-728.
- Phillips, A. T., & Mulfinger, L. M. Cyclic Adenosine 3', 5'-Monophosphate Levels in Pseudomonas putida and Pseudomonas aeruginosa During Induction and Carbon Catabolite Repression of Histidase Synthesis. Journal of Bacteriology, 1981, 145, 1286-1292.
- Piperino, J. R., & Oxender, D. L. Amino Acid-Binding Protein Released from Escherichia coli by Osmotic Shock. Journal of Biological Chemistry, 1966, 241, 5732-5734.
- Pont, D. G. Mannitol Transport in Pseudomonas aeruginosa. Unpublished Masters Thesis, University of Georgia, 1973.
- Romano, A. H., Voytek, A., & Bruskin, A. M. Energization of Glucose Transport by Pseudomonas fluorescens. Journal of Bacteriology, 1980, 142, 755-762.
- Rosen, B. P. Basic Amino Acid Transport in Escherichia coli. Journal of Biological Chemistry, 1971, 246, 3653-3662.

- Rosen, B. P. Basic Amino Acid Transport in Escherichia coli., II. Purification and Properties of an Arginine-Specific Binding Protein. Journal of Biological Chemistry, 1973, 248, 1211-1218.
- Rosen, B. P., & Vasington, F. D. Purification and Characterization of a Histidine-Binding Protein from Salmonella typhimurium LT-2 and Its Relationship to the Histidine Permease System. Journal of Biological Chemistry, 1971, 246, 5351-5360.
- Saier, M. H., Jr. Bacterial Phosphoenolpyruvate: Sugar Phosphotransferase Systems: Structural, Functional, and Evolutionary Interrelationships. Bacteriological Reviews, 1977, 41, 856-871.
- Shapiro, L., Agabian-Keshishian, N., Hirsh, A., & Rosen, O. M. Effect of Bibutyryladenine 3': 5'-Cyclic Monophosphate on Growth and Differentiation in Caulobacter crescentus. Proceedings of the National Academy of Sciences, 1972, 69, 1225-1229.
- Siegel, L. S., Hylemon, P. B., & Phibbs, P. V., Jr. Cyclic Adenosine 3', 5'-Monophosphate Levels and Activities of Adenylate Cyclase and Cyclic Adenosine 3', 5'-Monophosphate Phosphodiesterase in Pseudomonas and Bacteroides. Journal of Bacteriology, 1977, 129, 87-96.
- Smyth, P. F., & Clarke, P. H. Catabolite Repression of Pseudomonas aeruginosa Amidase: The Effect of Carbon Source on Amidase Synthesis, Journal of General Microbiology, 1975, 90, 81-90.
- Stinnett, J. D., Gilleland, H. E., Jr., & Eagon, R. G. Proteins Released from Cell Envelopes of Pseudomonas aeruginosa on Exposure to Ethylenediaminetetraacetate: Comparison with Dimethylformamide-Extractable Proteins. Journal of Bacteriology, 1973, 114, 399-407.
- Stinnett, J. D., Guymon, L. F., & Eagon, R. G. A Novel Technique for the Preparation of Transport-Active Membrane Vesicles from Pseudomonas aeruginosa: Observations on Gluconate Transport. Biochemical and Biophysical Research Communications, 1973, 52, 284-290.
- Stinson, M. W., Cohen, M. A., & Merrick, J. M. Isolation of Dicarboxylic Acid- and Glucose-Binding Proteins from Pseudomonas aeruginosa. Journal of Bacteriology, 1976, 128, 573-579.
- Stinson, M. W., Cohen, M. A., & Merrick, J. M. Purification and Properties of the Periplasmic Glucose-Binding Protein of Pseudomonas aeruginosa. Journal of Bacteriology, 1977, 131, 672-681.
- Taylor, R. T., Norrell, S. A., & Hanna, M. L. Uptake of Cyanocobalamin by Escherichia coli B: Some Characteristics and Evidence for a Binding Protein. Archives of Biochemistry and Biophysics, 1972, 148, 366-381.

- Tiwari, N. P., & Campbell, J. J. R. Enzymatic Control of the Metabolic Activity of Pseudomonas aeruginosa Grown in Glucose or Succinate Media. Biochimica et Biophysica Acta, 1969, 192, 395-401.
- Wasserman, R. H., Corradino, R. A., & Taylor, A. N. Vitamin D-Dependent Calcium-Binding Protein: Purification and Some Properties. Journal of Biological Chemistry, 1968, 243, 3978-3986.
- Weiner, J. H., & Heppel, L. A. A Binding Protein for Glutamine and Its Relation of Active Transport in Escherichia coli. Journal of Biological Chemistry, 1971, 246, 6933-6941.
- Wilson, D. B. Cellular Transport Mechanisms. Annual Review of Biochemistry, 1978, 47, 933-965.
- Wilson, O. H., & Holden, J. T. Arginine Transport and Metabolism in Osmotically Shocked and Unshocked Cells of Escherichia coli W. Journal of Biological Chemistry, 1969, 244, 2737-2742.
- Zwaig, N., & Lin, E. C. C. A Method for Isolating Mutants Resistant to Catabolite Repression. Biochemical and Biophysical Research Communication, 1966, 22, 414-418.