TRANSPORT AND CATABOLISM OF GLYCEROL IN *PSEUDOMONAS AERUGINOSA*

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CHAPTER I

INTRODUCTION

The regulation of gene expression is currently attracting considerable interest among scientists. Undoubtedly, the regulation of gene function in the more complex cells of higher organisms involves more complicated mechanisms than those used by bacteria. Nevertheless, studies of the control of metabolism in bacterial systems will provide useful models for the study of complex systems. If the bacterial models are to be of value, it is important that extrapolations to higher organisms be made from systems established as being of general occurrence in lower organisms (Demerec, 1964).

In 1961, Jacob and Monod (1961a, 1961b) proposed a new genetic unit, the operon, which is a cluster of linked structural genes and an operator which controls their expression. In examining the applicability of the operon concept to metabolic controls in any organism, it is important to obtain both biochemical evidence of the functioning of control systems and genetic data defining the relative locations of related genes. In several bacteria, some or all of the enzymes of a biosynthetic or degradative pathway are known to be produced from a group of clustered genes. Demerec (1964) has summarized the incidence of clustering of related genes as determined by transduction in Salmonella typhimurium. Demerec stated that about three-fourths of the gene loci among fourteen

groups of genes in this organism are closely linked; i.e., "a majority of the gene loci that represent phenotypically related groups are arranged in clusters." These fourteen groups included 87 genes. Clustering of related genes has been reported for the histidine pathway (Hartman, 1956), the tryptophan pathway (Blume and Balbinder, 1966), the leucine operon (Margolin, 1963), the arginine pathway (Demerec, et al., 1960), the methionine pathway (Smith, 1961), the purine pathway (Demerec, et al., 1956), the proline pathway (Miyake and Demerec, 1960), the pathways for aromatic amino acids and for pyrimidines (Demerec, 1964), the isoleucine-valine, threonine, serine and glycine pathways (Glanville and Demerec, 1960), and the pantothenic acid pathway (Demerec, et al., 1959). In Escherichia coli, the linkage of functionally related genes has been reported for the biosynthetic pathways for tryptophan (Yanofsky and Lennox, 1959), isoleucine-valine (Norväth, et al., 1964), the pyrimidines (Beckwith, et al., 1962), arginine (Gorini, et al., 1961), and diaminopimelic acid and methionine (Demerec, 1964), and for the degradative pathways for arabinose (Gross and Englesberg, 1959), lactose (Jacob, et al., 1960), and galactose (Lederberg, 1960). In Bacillus subtilis, linkage has been shown for the genes of the tryptophan pathway (Anagnostopoulos and Crawford, 1961), the pathway for aromatic biosynthesis (Nester, et al., 1963), and the histidine pathway (Ephrati-Elizur, et al., 1961).

In Neurospora crassa, of the many loci studied, linkage of related genes has been found only for some of the loci in the histidine biosynthetic pathway (Weber and Case, 1960), and in the aromatic pathway (Gross and Fein, 1960). In Saccharomyces cerevisiae, the genes of the histidine pathway are scattered (Fink, 1964).

In 1963, Holloway, et al. reported that they found no gene clustering in the following pathways in Pseudomonas aeruginosa: tryptophan, methionine, histidine, leucine, arginine, and isoleucine-valine. However, only one or two genes from each pathway were studied. Further study of additional mutants by Fargie and Holloway (1965) showed that only one pair of markers (threonine pathway) among 32 different loci for 14 biosynthetic pathways was linked. They concluded that linkage of functionally related markers is quite rare in P. aeruginosa. The pathway of pyrimidine biosynthesis in P. aeruginosa has been studied more thoroughly by Isaac and Holloway (1968). By using transduction and conjugation studies of 25 uracil-requiring mutants, they showed that four genes for pyrimidine biosynthetic enzymes were not linked to each other, although the pathway was the same as in E. coli, in which these genes had been found to be linked. Partial linkage of functionally related genes has been found in the pathway for tryptophan in P. putida (Crawford and Gunsalus, 1966), in the histidine pathway in P. aeruginosa (Mee and Lee, 1967), and in the methionine-cysteine pathway in P. aeruginosa (Calhoun and Feary, 1969). Thus, Pseudomonas has been reported to have a very different gene arrangement from that of E. coli or S. typhimurium. At the maximum, two or three related genes may apparently be linked, but no clustering of all the genes of any one pathway has been found. Thus, as pointed out by Demerec (1964), it is important that both genetic organization and the functioning of metabolic controls be studied in the genus Pseudomonas and compared to those in the enteric group.

Most of the genetic studies of Pseudomonas which have been done have involved biosynthetic pathways, and little attention has been paid

to the genetics of catabolic pathways. Glycerol was chosen for the present studies since utilization of glycerol in E. coli has been extensively studied by Lin and his coworkers, and, therefore, the comparison of genetic organization and control mechanisms for the same pathway in E. coli and P. aeruginosa is possible.

The major pathways for degradation of glycerol in bacteria are shown in Figure 1. In the first pathway, glycerol is initially phosphorylated to glycerophosphate before oxidation to dihydroxyacetone phosphate (reactions 1 and 2). This pathway has been shown in Mycobacterium smegmatis (Hunter, 1953), and Mycob. tuberculosis (Winder and Brennan, 1966), Streptococcus faecalis (Gunsalus and Umbreit, 1945; Jacobs and Van Demark, 1960), E. freundii (Mickelson and Shideman, 1947), Aerobacter aerogenes (Burton and Kaplan, 1953; Magasanik, et al., 1953), B. subtilis (Mindich, 1968), and E. coli (Koch, et al., 1964).

In the second pathway, glycerol is first oxidized to dihydroxyacetone, then phosphorylated to form dihydroxyacetone phosphate (reactions 3 and 4). This pathway has been found in A. aerogenes (Burton and Kaplan, 1953), B. subtilis (Wiame, et al., 1954), E. coli (Asnis and Brodie, 1953), Acetobacter suboxydans (Virtanen and Nordlund, 1933), Mycob. tuberculosis (Goldman, 1963), and S. faecalis (Gunsalus, 1947). The dihydroxyacetone phosphate formed in either case is then converted into glyceraldehyde-3-phosphate by triose phosphate isomerase (reaction 5) and proceeds through the usual glycolytic pathway (the Embden-Meyerhof-Parnas pathway).

A third pathway for glycerol catabolism has been proposed. This pathway has not been completely studied but it involves the transformation of glycerol into β-propionaldehyde, β-hydroxypropionic acid and

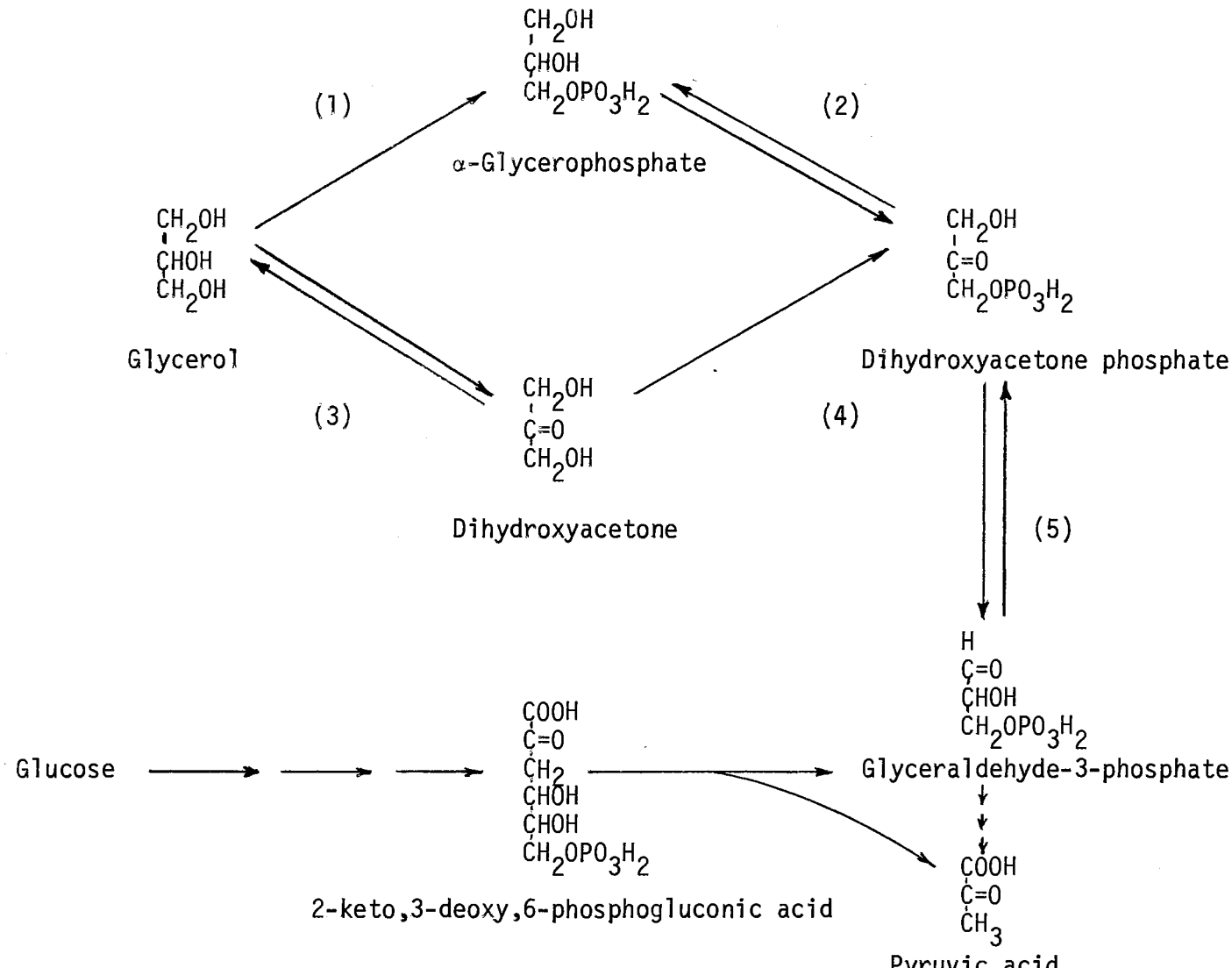


Figure 1. Major Pathways for Glycerol Metabolism in Bacteria.

trimethylglycol in lactobacilli (Cantoni and Molnar, 1967).

Both the first two pathways may be found in the same organism, e.g., in A. aerogenes strain 1033 (Lin, et al., 1960), B. subtilis strains 168 and B24 (Mindich, 1968; Wiame, et al., 1954), and S. faecalis strain 10C1 (Jacobs and Van Demark, 1960). In S. faecalis and A. aerogenes, the cells contain a flavin adenine dinucleotide (FAD)-linked or a nicotinamide adenine dinucleotide (NAD)-linked glycerol dehydrogenase, respectively, when grown on glycerol under anaerobic conditions. When exposed to aerobic conditions, the glycerol dehydrogenase is rapidly destroyed and glycerol is converted to glycerophosphate, followed by oxidation to triose phosphate by a glycerophosphate dehydrogenase which does not require NAD. In B. subtilis strains B24 and 168, the cells have an NAD-independent glycerophosphate dehydrogenase and a glycerol kinase for the first pathway (Mindich, 1968) and glycerol dehydrogenase and dihydroxyacetone kinase for the second pathway (Wiame, et al., 1954); however, the conditions of aeration for growth are not mentioned in these reports.

Different pathways have been described for glycerol catabolism in E. coli by different investigators. Most of the biochemical and genetic studies of utilization of glycerol in E. coli have been done by Lin and his coworkers. Asnis and Brodie (1953) reported the conversion of glycerol to dihydroxyacetone by extracts of E. coli, but the conditions of growth of the cells were not described. More recently, Koch, et al. (1964) studied the aerobic catabolism of glycerol in E. coli strain K 10. Glycerol kinase, L-α-glycerophosphate dehydrogenase, and L-α-glycerophosphate transport system were shown to be responsible for the dissimilation of glycerol and L-α-glycerol phosphate. These three

functional units can be induced by growth on either glycerol or L-α-glycerophosphate. All are very sensitive to catabolite repression exerted by glucose.

Further studies were directed toward determining the true inducer for these proteins. A mutant which produces a protein which has only slight enzymic activity but is immunochemically indistinguishable from crystallized glycerol kinase was isolated by Hayashi and Lin (1965b). In this mutant, the cross-reacting protein is inducible by L-α-glycerophosphate but not by glycerol. This finding allowed them to conclude that, in wild type cells, the induction of glycerol kinase by glycerol depends on the conversion of glycerol into its phosphorylated derivative by a small amount of kinase which is present in the uninduced cell. Experimental evidence that glycerophosphate dehydrogenase-negative mutants are hypersensitive to induction by L-α-glycerophosphate also supports the conclusion that L-α-glycerophosphate is the true inducer (Cozzarelli, et al., 1968).

Feedback inhibition plays a part in the regulation of biosynthetic pathways in bacteria. A case of feedback inhibition in a catabolic pathway has been reported in glycerol catabolism in E. coli. Utilization of glycerol was shown to be greatly reduced in the presence of glucose by Zwaig and Lin (1966). Individual intermediates of the glycolytic pathway were tested to identify the inhibitor and fructose-1,6-diphosphate was found to be inhibitory to glycerol kinase.

Further evidence for this regulatory role of fructose-1,6-diphosphate was reported by Böck and Neidhardt (1966a, 1966b) in their study of an E. coli mutant with a temperature-sensitive aldolase. This mutant was able to grow in rich medium (tryptone, yeast extract and

glucose) at 30 C but not at 40 C. Growth of the mutant at 40 C was inhibited by the presence of glucose in the medium. During glucose metabolism at 40 C, the level of fructose-1,6-diphosphate in the cell was more than 20-fold above normal. When glycerol was used to grow the mutant at 40 C, the addition of glucose to the medium caused cessation of glycerol metabolism. Growth on α-glycerophosphate was not inhibited under these conditions, suggesting glycerol kinase as a possible site of inhibition. Böck and Neidhardt concluded that the inhibitory effect of glucose on growth at 40 C might be the result of a generalized poisoning of many cell processes by a greatly increased intracellular concentration of fructose-1,6-diphosphate (sugar phosphate toxicity).

Zwaig, et al. (1970) found a mutant strain which produced a glycerol kinase resistant to inhibition by fructose-1,6-diphosphate. This mutant grew faster than its wild type parent on glycerol. The alteration in glycerol kinase of the mutant strain, which decreased the sensitivity to feedback inhibition, did not eliminate catabolite repression of the glycerol pathway in the presence of glucose. However, if the altered enzyme was produced constitutively, diphasic growth did not occur. The desensitization of glycerol kinase to feedback inhibition allows glycerol to exert catabolite repression on glycerol enzymes and also on lactose enzymes. The cells with a high level of kinase activity are subject to toxicity and death due to overproduction of glycerophosphate if the supply of glycerol is suddenly increased.

Glycerol kinase, the L-α-glycerophosphate transport system and L-α-glycerophosphate dehydrogenase can be rendered constitutive simultaneously by an apparently single mutation. This suggested that the structural genes for the three units could belong to the same operon or

to different operons which share the same regulator gene. Experimental data which showed different sensitivities of the three units to catabolite repression in a mutant constitutive for all three favored the latter possibility (Koch, et al., 1964).

Recent experiments by Cozzarelli, et al. (1968) showed that these three units are coded by genes widely spaced on the genome and are controlled by a single regulator gene which is close to (possibly contiguous to) the structural gene for L-α-glycerophosphate dehydrogenase. A mutation of the regulator gene can cause constitutivity, non-inducibility or temperature sensitivity of all three units. Deletion of this gene causes the derepression of all three operons, i.e., "the L-α-glycerophosphate system is a regulon under negative control."

The catabolism of glycerol in P. aeruginosa strain 1 (PA-1) has been studied in this laboratory by Cowen (1968). He showed, by assays of enzymes in extracts, that the pathway involved first the phosphorylation of glycerol by glycerol kinase, and then oxidation by an NAD-independent glycerophosphate dehydrogenase. This pathway is the same as that found in the aerobic dissimilation of glycerol in A. aerogenes 1041 and E. coli K 10. The NAD-linked glycerol dehydrogenase of the anaerobic pathway in these organisms may be considered absent in strain PA-1, since no reduction of NAD was observed in extracts of fully induced cells. Neither NAD-linked glycerol dehydrogenase nor NAD-linked glycerophosphate dehydrogenase was found when NAD reduction capacity was measured in cell free extracts with the addition of glycerol and glycerophosphate. Glycerol kinase and L-α-glycerophosphate dehydrogenase were shown to require induction with glycerol, by testing the enzyme activity of extracts of cells grown on glucose and glycerol individually.

Variation in levels of triose phosphate isomerase, required for the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate, was not considered significant for glycerol or glucose-grown cells since both had very high isomerase activity. No growth of strain PA-1 was found on L-α-glycerophosphate minimal medium; this may suggest that a transport system for L-α-glycerophosphate is lacking in PA-1. The catabolism of glycerol was found to be subject to inhibition in the presence of glucose.

Two groups of glycerol-negative mutants were isolated by Cowen (1968). One group (Group I) could grow on glucose minimal medium. A second group of mutants (Group II) not only could not use glycerol as carbon source, but also could not grow on mannitol. Growth on glucose required the addition of yeast extract.

Further study by Mose (1970) showed that the growth factor required for growth on glucose is methionine. Mose found that these mutants also could not utilize gluconate or glutamic acid as a carbon source even with the addition of methionine.

Enzyme studies by Cowen (1968), using one mutant from each group, showed no apparent loss of glycerol kinase activity in either mutant. L-α-glycerophosphate dehydrogenase activity was present in the mutant from Group I, but was missing in the Group II mutant. Triose phosphate isomerase activity was greatly reduced in both mutants; however, a basal level was present in both mutant extracts.

The susceptibility to phage was different in the two sets of mutants. Group I cells were sensitive to Pseudomonas phage Pø-6 and Group II cells were resistant to this phage and 12 other phages tested by Cowen. Further studies by Mose (1970) showed that each of the mutants of Group

II was resistant to infection by 34 phages capable of lysing the wild type parent. Mutants of Group I had a high reversion rate, which suggested that these cells may have only single site mutations. Mutants of Group II are quite interesting. These mutants may have resulted from deletions since no revertants have been found for either the glycerol or methionine locus. The loss of ability to utilize glycerol and to synthesize methionine and loss of sensitivity to phage may be explained in two ways. First, the mutation could be found in a change in the cell surface which affects both phage adsorption and the conformation of surface-related enzymes, as suggested for proline deficiency in T_4-resistant mutants of E. coli (Baich, 1968). Second, the simultaneous mutation of three loci may be the result of deletions extending into adjacent loci, i.e., at least one glycerol locus, one methionine locus and a locus involved in the structure of a phage adsorption site may be closely linked. Cozzarelli and Lin (1966) showed that loci for methionine biosynthesis and glycerol kinase are closely linked in E. coli. Another group of mutants isolated by C. C. Green (1969) for methionine auxotrophy also are phage resistant and are unable to use glycerol (Mose, 1970). Therefore, the deletion hypothesis has been considered the more probable of the two (Mose, 1970). However, data obtained in the present investigation may modify this conclusion.

Proteins involved in the transport of substrates into the bacterial cell may be synthesized coordinately with the catabolic enzymes; i.e., the locus specifying a transport protein may be included in the operon. In other cases, the locus for transport may be unlinked to loci for the catabolic enzymes but may still be subject to the same controls (Epstein and Beckwith, 1968). Thus, in the study of a catabolic pathway and its

control, it is important to determine the mechanism by which the substrate enters the cell and to determine whether entry is subject to the same controls as are the catabolic enzymes.

The transport of molecules across cell membranes has been attributed to four mechanisms: simple diffusion, facilitated diffusion, an active transport system and a bacterial phosphotransferase system. In simple diffusion, the molecule can cross the cell membrane freely in the absence of a membrane carrier. Water and other small molecules can penetrate into the cell by this mechanism. In facilitated diffusion, a specific membrane carrier moves the molecule across the cell membrane without energy coupling, catalyzing the rapid equilibration of external and internal concentrations. Larger molecules and some ions require a specific membrane protein with or without energy coupling for transport into the cell. Active transport involves energy coupling with a membrane carrier. A general model for transport systems was suggested by Pardee (1967b). The model assumes that the first step is recognition, in which the molecule combines with an active site on the membrane surface; the second step is translocation, in which the molecule is carried across the membrane; the third step is release of the molecule inside the cell; and the fourth is recovery of the transport system to its original state. The requirement for energy may be related to any or all of the last three steps. Facilitated diffusion and active transport differ in two major respects: an energy input is required in the case of active transport, and facilitated diffusion does not concentrate compounds against a gradient as does active transport.

Another type of energy-coupled transport system is the phosphotransferase system in bacteria which thus far has been shown to function

especially for sugar transport. Phosphoenolpyruvate (PEP) is the high-energy phosphate donor. Kundig, et al. (1964) discovered an energy-supplying heat-stable protein, HPr. This protein is phosphorylated by PEP in a reaction mediated by "Enzyme I" of this transport system. The phosphorylated HPr then acts as phosphate donor for the sugar in a reaction mediated by "Enzyme II." HPr and Enzyme I are not specific for each individual sugar and are formed constitutively. However, Enzyme II, the component tightly bound to the membrane, is specific for each sugar, and most of the Enzymes II are inducible. In this transport system sugars are accumulated as their phosphorylated derivatives or the original sugar is formed by dephosphorylation inside the cell if the phosphorylated product is not utilizable by cellular enzymes (Kaback, 1970). Phibbs and Eagon (1970) studied the transport and phosphorylation of glucose, fructose and mannitol by P. aeruginosa. They concluded that carbohydrates are not transported into P. aeruginosa through a phosphoenolpyruvate transferase system. Rather, the carbohydrates are transported into the cells, then phosphorylated via specific kinases to form sugar phosphate, using ATP as the phosphate donor.

Neu and Heppel (1965) and Nossal and Heppel (1966) showed that cells osmotically shocked by treatment with ethylenediaminetetraacetate, sucrose and Tris release a group of enzymes and/or proteins associated with the cell envelope (presumably from the pericytoplasmic space) without destroying their activities. Piperno and Oxender (1966) found that the transport activities for leucine and valine were reduced in cells osmotically shocked at 0 C; however, binding activity for these amino acids was found in the lyophilized supernatant, or "shock fluid."

The characteristics of transport systems have been summarized as

follows (Pardee, 1968): first, transport systems may be protein because of their specificity, which resembles that of an enzyme for its substrate. Second, the initial entry rate of a substrate depends on its concentration, indicating a limited number of independent adsorption sites. Third, a carrier molecule can apparently cycle between inward and outward states, as indicated by ability of a compound to stimulate exit of a similar compound. Fourth, active transport is inhibited by reagents which react with proteins and synthesis of transport systems is prevented by inhibitors of protein synthesis. Therefore, we can conclude that protein must be at least the major component of the binding site for transport. Purification and crystallization of transport proteins are the next important step in studies of the binding component. Purified binding proteins have been isolated by Pardee (1966, 1967a) from S. typhimuriun and by Anraku (1968), and Penrose, et al. (1968) from E. coli.

The transport of glycerol and L-α-glycerophosphate has been studied to some extent in E. coli. Hayashi and Lin (1965a) showed that mutant cells lacking glycerol kinase were unable to accumulate radioactivity from glycerol-^{14}C or to grow on glycerol. They also found that free diffusion of glycerol into cells of E. coli was not rate-limiting for growth, even at very low concentrations, and concluded that this was the mode of entry of glycerol in E. coli. Its retention in the cell was said to be due to its conversion to glycerophosphate. No active transport system was found for glycerol. Further studies, however, showed that glycerol entered the cell by facilitated diffusion (Sanno, et al., 1968). Induction by glycerol or glycerophosphate was required for the entry of glycerol. No induction was required for a constitutive mutant.

These results indicated that the entry of glycerol into E. coli is not by simple diffusion, as was originally thought, but is controlled by a specific gene product. L-α-glycerophosphate was found to be taken up by E. coli cells through an active transport system (Hayashi, et al., 1965), and no prior hydrolysis to free glycerol was required for the utilization of L-α-glycerophosphate (Lin, et al., 1962).

The present investigation was undertaken to extend previous studies in this laboratory on glycerol catabolism, to examine the nature of the true inducer, to study the glucose effect, and to examine the transport system for glycerol in P. aeruginosa. Isolation of a glycerol kinase-less mutant is required in order to determine with certainty whether glycerol or glycerol phosphate is the true inducer. Therefore, attempts were made to isolate such a mutant. Measurements of enzyme activities in cell-free extracts of wild type and mutant cells grown under different conditions were used to investigate control of enzyme synthesis. Transport of glycerol was studied using labeled and unlabeled glycerol in induced and uninduced cells to define the type of transport system used in this organism for glycerol. Shock fluid and binding protein were also prepared and used to study glycerol transport. Equilibrium dialysis was used for detection of binding activity. An additional objective of the present study was to obtain glycerol mutants sufficiently stable to be used in mapping the loci for the glycerol enzymes in relation to each other and to the loci for phage resistance and methionine biosynthesis.

CHAPTER II

MATERIALS AND METHODS

A. Bacteria and Bacteriophages

Strain 1 of P. aeruginosa (designated as PA-1 in the text) and the mutants derived from it were used throughout these studies. Strain PA-1 was originally obtained from B. W. Holloway of the University of Melbourne. Mutants numbered PA-1-92 through PA-1-97 were obtained from C. Green (1969). They were initially selected as methionine auxotrophs after a 16-hour treatment with ethyl methane sulfonate (Necasek, et al., 1967). Mutants PA-1-623, PA-1-652 and PA-1-663 were isolated by treatment of PA-1 with nitrosoguanidine (Cowen, 1968). These were originally selected for inability to grow on glycerol. Mutants PA-1-27, PA-1-55, and PA-1-64 were isolated in this laboratory by James Cowley after three successive treatments of PA-1 with ethyl methane sulfonate and enrichment with penicillin, using the method described by Bruce (1965). They were originally selected as methionine auxotrophs.

Bacteriophage Pø-6, used for isolation of phage-resistant mutants, and phage Pø-5, used for transduction, were obtained from R. Green in this laboratory. Phage F116, used for checking phage sensitivity, was originally isolated by Dr. B. W. Holloway.

B. Media

The medium used for growth of bacteria in the majority of

experiments was "Pseudomonas minimal medium" which was a modification of M-9 medium (Roberts, et al., 1957), and contained the following components (amounts per liter): $Na_2HPO_4 \cdot 7H_2O$, 8.2 gm; KH_2PO_4, 2.7 gm; $MgSO_4 \cdot 7H_2O$, 0.4 gm; NH_4Cl, 1.0 gm; $FeSO_4$, 0.1% solution, 0.5 ml; distilled water to volume. Two per cent agar was added for the preparation of plates. The $MgSO_4 \cdot 7H_2O$ was autoclaved separately as an 8 per cent (w/v) solution and added to the sterile medium to the desired concentration. Carbon sources were sterilized separately by autoclaving as 10 per cent solutions (w/v) and added to the desired concentration as required. Methionine was prepared by filter sterilization as a 0.2 per cent solution (w/v).

Pseudomonas phage broth was used for storage of phage suspensions and for making dilutions of phage stocks. It was composed of (gm per liter): Difco nutrient broth, 8.0; Difco yeast extract, 5.0; NaCl, 5.0; distilled water to volume. For platings, the bottom layer contained 1.1 per cent of Difco agar per liter of broth; plates contained 35 ml of agar. The top layer contained 0.65 per cent agar per liter of broth (Holloway, et al., 1962).

C. Growth of Bacteria

Growth of all cultures was at 37 C and liquid cultures were incubated on a reciprocal shaker. Growth was measured as optical density at 540 nm. The cell suspensions were read against appropriate blanks in 18 mm tubes in a Coleman Junior Spectrophotometer, Model 6-D.

D. Preparation and Titration of Phage Plate Stocks

Phage plate stocks used for isolation of resistant bacterial mutants, for testing cultures for sensitivity to phage and for attempted

transductions were prepared according to a modification of the method of Swanstrom and Adams (1951). One drop of log phase cells and 0.1 ml of a phage suspension containing approximately 5 x 10^6 plaque-forming units (PFU) per ml were added to 3.0 ml of Pseudomonas phage agar (top layer), mixed, and poured over 35 ml of bottom-layer Pseudomonas phage agar. The plates were incubated at 37 C for 12 hours, and the phage were soaked off by flooding the plate with 5.0 ml of Pseudomonas phage broth. After 30 minutes, the broth was pipetted from the plates and the cells were removed by centrifugation. The supernatant was filtered through a Millipore filter (HA, 0.45 μ pore size).

For determining the titer of phage suspensions, a 0.1 ml volume of appropriately diluted phage suspension was added to 3.0 ml of top layer phage agar along with one drop of a suspension of the appropriate strain of bacteria. This was mixed and poured over the bottom layer. After the agar had solidified, plates were incubated at 37 C. Plaques were counted after approximately 12 hours.

E. Isolation of Glycerol Mutants

1. Selection of Mutants Using Nitrosoguanidine and Antibiotics

The procedure used in isolation of mutants was a modification of the method of Adelberg, et al. (1965). PA-1 was inoculated into 6 ml of nutrient broth and grown to approximately 10^9 cells per ml. The cells were centrifuged, washed with 0.85 per cent (w/v) NaCl, resuspended in 6 ml of 0.05 M, pH 6.0, acetate buffer which contained nitrosoguanidine at 100 μg per ml, and incubated 30 minutes on a shaker at 37 C. The cells were collected by centrifugation, washed with saline, divided into 10 separate tubes, each containing 6 ml of 0.5 per cent glucose

minimal medium, and allowed to grow to stationary phase. The cells were harvested by centrifugation and washed with saline. Tubes were prepared which contained 0.5 per cent glycerol and 50 μg per ml of carbenicillin in minimal medium in a total volume of 6 ml. Cells from the saline suspensions were added to the carbenicillin medium to a concentration of 10^6 cells per ml and incubated for 12 hours. A series of dilutions was plated on glucose minimal agar to determine the proper dilution to use to obtain approximately 100 colonies per plate. Five plates of the appropriate dilution from each tube were spread and incubated until small colonies had appeared. The glucose plates were replicated onto glucose and glycerol minimal agar. Colonies which grew well on the glucose medium, but slightly or not at all on glycerol, were selected for further study. Other experiments were similar to that above except that the enrichment step was changed. Instead of carbenicillin, 650 μg per ml of D-cycloserine was added to the glycerol minimal medium for mutant selection.

2. Selection of Mutants from Phage-resistant Colonies

Cells grown in glucose minimal medium to exponential phase were centrifuged, washed with saline, resuspended and diluted to between 10^4 and 10^5 cells per ml in glucose minimal medium. Acriflavin was added to this cell suspension to a concentration of 2.5 μg per ml. The tubes were incubated on a 37 C shaker until growth reached the stationary phase. The cells were harvested by centrifugation and washed with saline. Tubes were prepared to contain carbenicillin and cells in glycerol minimal medium as described in Section E, 1, above, and incubated for 12 hours at 37 C with shaking. One tenth ml of acriflavin-treated cells was transferred to 6 ml of nutrient broth, and allowed to

grow to exponential phase. One drop of this cell suspension and 0.1 ml of a 10^{-1} dilution of plate stock with a concentration of 1.0×10^{10} PFU/ml of phage Pø-6 were added to 3 ml of soft agar. This was mixed and poured over an agar layer as described for preparation of plate stocks. Plates were incubated at 37 C until colonies appeared. All of the colonies on the phage plates were picked off and each one was spot-tested on glucose and glycerol minimal plates. Colonies able to grow on glucose medium, but not on glycerol, were selected for further study.

F. Chemicals

D-glyceraldehyde-3-phosphate (diethyl-acetal, barium salt), glycerophosphate (α, β mixture disodium pentahydrate), muscle α-glycerophosphate dehydrogenase, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and L-methionine were obtained from Calbiochem. Glucose was from Baker Chemical Co., and glycerol from Fisher Scientific Co. Carbenicillin (α-carboxybenzylpenicillin) was a gift from Beecham Pharmaceuticals. D-cycloserine was obtained from Mann Research Laboratories, and acriflavin from Pfaltz and Bauer, Inc. Thiazolyl blue (MTT) [3(4,5 dimethyl thiazolyl 1-2) 2,5 diphenyl tetrazolium bromide] was a product of Nutritional Biochemicals Corporation. Glycerol-1,3-^{14}C was obtained from Nuclear-Chicago Corporation. N-methyl-N'-nitro-N-nitrosoguanidine was from Aldrich Chemical Company. Glucostat reagents were obtained from Worthington Biochemical Corporation. Nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH) were from Calbiochem or C. F. Boehringer. Adenosine triphosphate (ATP) was a product of Pabst Laboratores or Mann Research Laboratories.

G. Chemical Analyses

1. Glucose

To 9 ml of prepared Glucostat reagent, a one-ml sample containing 0.05 to 0.3 mg glucose was added and the tube was allowed to stand at room temperature for exactly 10 minutes. One drop of 4 M HCl was added to stop the reaction and stabilize color. Tubes were allowed to stand for five minutes after stopping the reaction, and absorbance was read on a Coleman Junior Spectrophotometer at 400 nm.

2. Protein

The method of Sutherland, et al. (1949) was used to determine the protein content of cell extracts. The extracts were diluted 1:20 and aliquots made up to 1.0 ml with water. Five ml of freshly-prepared reagent containing 100 ml of 4 per cent sodium carbonate, 1.0 ml of 4 per cent sodium-potassium tartrate and 1.0 ml of 2 per cent $CuSO_4$ were added to the protein sample. The tubes were allowed to stand for 40 minutes at room temperature. Phenol reagent, 2N (Fisher Scientific Company) was diluted 1:2 with water; 0.5 ml was added to each tube and mixed immediately. The color was read at 660 nm. Gregory and Sajdera (1970) reported that some buffers affect color production from protein and may even produce false color in the absence of protein; therefore, all protein concentrations for cell-free extracts were calculated based on a protein standard solution made up in the 0.1 M bicine buffer, pH 8.5, which was used to make cell-free extracts.

H. Preparation of Whole Cells for Assay of L-α-Glycerophosphate Dehydrogenase

Cowen's method (1968) was used for preparation of whole cells for

assay of α-glycerophosphate dehydrogenase. Forty ml of 0.2 per cent glucose medium were inoculated with 0.1 ml of a saline suspension of cells from a fresh nutrient agar slant. The culture was incubated on the shaker to late log phase, then divided into two 20-ml portions. To one portion of cells (A), glycerol was added to a concentration of 1.0 per cent. This portion of the culture was returned to the shaker and incubated for 4 hours more. The remaining cells (B) were centrifuged, rinsed with distilled water, resuspended in 20 ml of distilled water, and placed in an ice bath for 4 hours. They were then removed from the water by centrifugation and the pellet was frozen. The glycerol-treated cells were removed from the shaker, suspended in distilled water, and treated in the same way described for "B."

I. Preparation of Cell-free Extracts

Cells were grown in 900 ml of 0.5 per cent glucose minimal medium (methionine was added as required to a final concentration of 35 µg per ml). Cells were harvested by centrifugation during late log phase, washed with 0.85 per cent NaCl, resuspended in 1.0 per cent glycerol medium and incubated on the shaker at 37 C for 4 hours. The cells were collected by centrifugation, washed with saline, and frozen. The pellets were thawed. Cells were broken with a Bronson sonifier with intermittent cooling in ice after suspending in 0.1 M bicine [N,N-bis (2-hydroxyethyl) glycine] buffer at pH 8.5. Whole cells and cell debris were removed by centrifugation. Extracts were dialyzed against 4 liters of 0.02 M bicine buffer, pH 8.5, prior to use in enzyme assays.

J. Enzyme Assays

1. Glycerol Kinase

The method of Lin, et al. (1962) was used to measure the phosphorylation of glycerol by coupling with the L-α-glycerophosphate dehydrogenase reaction. The substrate was omitted in the blank. The assay tube contained: 0.3 ml of 0.1 M glycerol, 0.3 ml of 3 N hydrazine, 0.5 ml of 1.0 M sodium carbonate buffer at pH 9.5, 0.2 ml of 0.1 M ATP, 0.6 ml of 0.1 M $MgCl_2$, 0.2 ml of 0.02 M NAD, 0.3 mg L-α-glycerophosphate dehydrogenase (rabbit muscle) and cell-free extract in a total volume of 3.0 ml. NADH formation was measured at 340 nm in a Cary Recording Spectrophotometer, Model 14 (Applied Physics Corporation).

2. L-α-Glycerophosphate Dehydrogenase in Whole Cells

Cowen's (1968) method was used to measure the L-α-glycerophosphate dehydrogenase activity of whole cells by comparing the rate of dye reduction by induced and non-induced cell preparations on addition of substrate. The frozen cells were thawed by suspending them in 2.0 ml of 0.1 M phosphate buffer, pH 7.5; 0.2 ml of this cell suspension was added to a solution which contained 1.6 ml of 0.1 M phosphate buffer, pH 7.5, 0.2 ml of 0.15 M KCN and 0.1 ml of MTT (1 mg/ml). The endogenous rate of dye reduction was determined by reading optical density at 550 nm at one-minute intervals for 6 minutes using a Coleman Junior Spectrophotometer at room temperature. After 6 minutes, 0.2 ml of 1.0 M DL-α-glycerophosphate was added to both the induced and non-induced preparations. The optical density was recorded at one-minute intervals for an additional period of 8 to 10 minutes.

3. L-α-Glycerophosphate Dehydrogenase in Cell-free Extracts

L-α-glycerophosphate dehydrogenase was assayed by measuring the rate of reduction of the tetrazolium dye, MTT (thiazolyl blue) to its

formazan according to the procedure described by Lin, et al. (1962). Addition of KCN which prevented the passage of electrons through the cytochrome system, enhanced the measurement of dehydrogenase activity. The assay tube contained: 0.1 ml of 1.0 M DL-glycerophosphate (α, β), 0.1 ml of MTT (1 mg/ml), 0.2 ml of 0.15 M KCN, cell-free extract, and 0.1 M phosphate buffer, pH 7.5, to a final volume of 3.1 ml. The reaction was recorded on the Cary 14 recording Spectrophotometer at 550 nm.

4. Triose-phosphate Isomerase

Triose-phosphate isomerase activity was determined by coupling with added glycerophosphate dehydrogenase and measuring oxidation of NADH on the addition of DL-glyceraldehyde-3-phosphate according to Cowen (1968). The reaction mixture contained: 0.2 ml of 0.02 M NADH, cell-free extract, 0.3 mg L-α-glycerophosphate dehydrogenase (rabbit muscle), 0.5 ml of 0.025 M DL-glyceraldehyde-3-phosphate and 0.1 M bicine, pH 8.5, to a total volume of 3.7 ml. The substrate was replaced with water in the blank. Oxidation of NADH was followed at 340 nm using a Cary Spectrophotometer, by reversing the positions of cuvettes containing blank and sample.

K. Transduction

The transduction technique of Murphy and Rosenblum (1964) for Staphylococcus aureus was used with modifications. The plating medium was minimal salts containing either glucose or glycerol plus methionine. Cells were washed from a fresh nutrient agar slant with 2 ml of 0.85 per cent saline and 0.1 ml of this suspension was spread on the agar plate. One drop of phage Pø-5 plate stock was placed on the surface of the agar within a marked area and allowed to dry prior to inverting and incubating

the plate at 37 C. Plates were observed for transductants after 3-5 days.

L. Uptake of Glycerol

1. Measurement by Osmotic Effect

The method of Sanno, et al. (1968) was used to test for the entry of glycerol into the cell by a process other than simple diffusion. Cells grown with various carbon sources were harvested by centrifugation, washed once with 0.02 M cold Tris buffer at pH 7.5 and resuspended in 4 nM Tris buffer, pH 7.5, at an optical density of approximately 1.5. To 2.5 ml of this suspension in a cuvette (1 cm light path), placed in the cell compartment of the Cary 14 recording spectrophotometer, 0.5 ml of 4.8 M glycerol was rapidly injected through a hypodermic needle. Recording of the change in optical density of the suspension was initiated immediately. For these experiments cells were grown on nutrient broth or on minimal medium containing one of the following: 1.0 per cent glucose, 1.0 per cent glycerol, 0.5 per cent each glucose and glycerol, 1.0 per cent sodium lactate, 0.08 M α,β-glycerophosphate plus 1.0 per cent casamino acids (Difco), or 1.0 per cent casamino acids.

2. Measurement Using ^{14}C-Glycerol

The uptake of ^{14}C-labeled glycerol was measured by a modification (Brown, personal communication) of the method described by Eagon and Asbell (1969). Cells were grown in 100 ml of 0.5 per cent glucose minimal medium (methionine was added as required), collected by centrifugation during log phase, washed with 0.85 per cent saline and induced in 0.5 per cent glycerol minimal medium for 4 hours. The cells were centrifuged, washed with saline and resuspended in Pseudomonas minimal

medium to a final concentration of 1.5×10^9 cells per ml. Twenty-five ml of such a cell suspension was placed in a 250 ml Erlenmeyer flask on a reciprocal, water bath shaker at a speed of 135 cycles per minute and temperature of 37 C. At zero time, 100 µl of 159 µM ^{14}C glycerol (specific radioactivity 14.3 mc/mM) was added to the flask. A 1.0 ml sample was removed from the flask with a syringe and filtered through a Millipore filter (HA, 0.45 µ pore size) at the time intervals described for each experiment. The filter pads were washed twice with 1 ml of ice-cold 0.01 per cent unlabeled glucose minimal medium, placed in scintillation vials and immediately dried in a stream of hot air. Filter pads were broken up with a wooden stirring rod and to each vial were added 10 ml of ethyl alcohol-toluene (4:6) standard scintillation fluid containing 0.4 per cent 2,5-diphenyloxazole (PPO) and 0.02 per cent 1,4-bis [2-(5-phenyloxazolyl) benzene] (POPOP). Counting was done with a Nuclear-Chicago liquid scintillation counter, Model 720. Glucose grown cells were used to test for uptake by uninduced cells in the same way. An uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, and N-ethylmaleimide, which has been shown to inhibit binding of β-galactosides (Fox and Kennedy, 1965) were added to the cells to determine whether either would prevent glycerol uptake.

Most of the uptake data were corrected for quenching by calculating the counting efficiency using two different channels. Three sets of experimental data, those for glucose-grown cells, for PA-1-623 mutant cells and for osmotically shocked sphaeroplasts, were not corrected for quenching, because one of the channels was not available for use at the time.

The transport data are reported as n moles per mg dry weight of

cells. The cellular dry weight was calculated based on a dry weight for a single cell of 2×10^{-13} g. Therefore, 1.5×10^{9} cells per ml of cell suspension used for the experiment was equal to 0.3 mg dry weight of cells per ml.

M. Measurement of Binding Protein

1. Preparation of Shock Fluid

The glycerol binding activity in a cell-free preparation was measured using the following method (K. K. Brown, personal communication). Cells were grown in 1 liter of 0.5 per cent glycerol minimal medium at 37 C on a reciprocal shaker. The late log phase or early stationary phase cells were harvested by centrifugation and washed twice with 0.05 M Tris buffer, pH 7.0, at room temperature. The cells were resuspended in 15 ml of cold 0.5 M sucrose-1 mM ethylenediaminetetraacetate (EDTA) - 0.05 M Tris HCl, pH 8.0. The suspension was allowed to remain at room temperature for 20 minutes.

Sphaeroplast formation was checked by observing the lysis of a sample of the suspension in water. The suspension was centrifuged in the cold. The supernatant (shock fluid) was tested for glycerol binding activity. The protein of the shock fluid was also concentrated by adding solid $(NH_4)_2SO_4$ to 85 per cent saturation and collecting the precipitate by centrifugation. The pellet, resuspended in glass distilled water, was tested for binding activity.

2. Binding of ^{14}C-Glycerol by Shock Fluid

The binding activity of shock fluid and crude shock protein was studied by dialysis against labeled glycerol using an equilibrium dialysis cell (Chemical Rubber Co.). One ml of shock fluid or crude

shock protein was placed on one side of the cell and 1 ml of 1.6 mM ^{14}C-glycerol was added to the other side of the cell. The two halves of the cell were separated by cellulose dialysis tubing, 1 1/8-inch inflated diameter, which had been boiled in 1 mM EDTA to remove metal ions. The cells were placed on a shaker over night at room temperature. Inactivation of binding activity was studied in the same way, using shock fluid which had been heated for 5 min at 70 C or shock fluid treated with 1 mM N-ethylmaleimide and dialyzed against labeled glycerol.

3. Analysis of Radioactive Compounds Accumulated from ^{14}C-Glycerol

After exposing cells to ^{14}C-glycerol under conditions described in the previous section, uptake was stopped by pipetting 1 ml of the cell suspension onto a Millipore filter and washing twice with cold 0.1 M KCN (Villarreal-Moguel and Ruiz-Herrera, 1969). The filter pad was placed in a tube, 10 ml of water were added and the tube was agitated vigorously. The tube was then heated in a boiling water bath for 5 min and again agitated vigorously. The filter pad was removed and the suspension was lyophilized and resuspended in 150 µl of H_2O. A 100 µl sample was spotted on Whatman No. 4 chromatography paper which had been prewashed with distilled water-pyridine-glacial acetic acid (80:15:15, v/v/v). The chromatograms were then developed (descending) with a solvent mixture containing isopropyl alcohol, pyridine, glacial acetic acid, distilled water in the volume ratio 8:8:1:4 (Gordon, et al., 1956) for about 11 hours at room temperature. Labeled and unlabeled glycerol, and unlabeled L-α,β-glycerophosphate and dihydroxyacetone phosphate standards were also chromatographed. The developed chromatograph strips were then passed through a Packard Radiochromatogram Scanner, Model 7201 and the relative positions of the accumulated radioactive materials were

compared to those of the standards. A sample prepared as described above was hydrolyzed with 2 N HCl in a boiling water bath for 1.5 hours, lyophilized and spotted on paper for checking products formed by hydrolysis of possible complex compounds.

Carbohydrates were detected by dipping the paper strips through a solution containing 0.005 M periodic acid in acetone. The strip was allowed to dry in air at room temperature for 3 to 4 minutes, dipped into 0.01 M benzidine solution and again allowed to dry. As it dried, the background became deep blue with white or yellow spots. After 5 to 10 minutes, when the spots showed maximum contrast, they were outlined in pencil. The background color slowly faded to gray and the spots to grayish-white (Gordon, et al. 1956).

CHAPTER III

EXPERIMENTAL RESULTS

A. Control of Glycerol Catabolism in Pseudomonas aeruginosa

Extracts were prepared from wild type (PA-1) cells grown or induced with a number of different carbon sources, and assays of the glycerol-degrading enzymes were carried out in order to determine the effect of these carbon sources on enzyme levels. Glycerol kinase and L-α-glycerophosphate dehydrogenase activities were determined in all extracts since these two enzymes are specific for glycerol metabolism. Triose phosphate isomerase activity is necessary for metabolism of glycerol but it is also required for metabolism of all other carbon sources which utilize the EMP pathway for either degradation or synthesis. This activity was measured in extracts of cells treated in four different ways. The enzyme assay data are summarized in Table I.

Two carbon sources, glucose and lactate, were used as substrates for growth of uninduced cells. In lactate-grown cells no kinase or dehydrogenase activity was detected, while very low levels of both enzymes were present in extracts of glucose-grown cells. Triose phosphate isomerase activity in glucose-grown cells was found to be more than 1000-fold higher than that of the enzymes specific for glycerol. Isomerase activity was also quite high in cells grown on lactate or nutrient broth or induced with glycerol after growth on glucose.

TABLE I

SPECIFIC ACTIVITIES OF GLYCEROL ENZYMES IN CELL-FREE EXTRACTS OF WILD TYPE CELLS

Preparation of Cells	Specific Activities (n moles/min/mg protein)		
	Kinase	Dehydrogenase	Isomerase
Grown on lactate	0	0	424
Grown on glucose	0.37	0.14	705
Induced with glycerol	5.55*	5.69*	393
Grown on nutrient broth	1.43*	0.93*	158
Grown on nutrient broth with glycerophosphate	1.75	2.12	
Grown on glucose plus glycerol	6.39	4.14	
Induced with glucose plus glycerol	4.12	2.67	

*The data for these enzyme assays are the averages of determinations using two different cell extracts.

The inducibility of the kinase and dehydrogenase was shown by preparing extracts from cells exposed to glycerol for four hours after growth on glucose. Kinase activity was increased 15-fold and dehydrogenase activity was increased 40-fold by induction with glycerol. Isomerase activity was decreased after exposure to glycerol.

Since Hayashi and Lin (1965b) have reported that L-α-glycerophosphate is the true inducer of the glycerol-degrading enzymes of E. coli, its ability to act as inducer in P. aeruginosa was determined. P. aeruginosa, unlike E. coli, cannot use glycerophosphate as a carbon source (Cowen, 1968), so it was added to nutrient broth, cells were grown in the mixture and extract was prepared. Enzyme activities were determined in extract from cells grown on nutrient broth alone as a control. Levels of both kinase and dehydrogenase were somewhat higher in nutrient broth-grown cells than in cells grown on lactate or glucose. This may indicate that glycerol-containing lipids or other glycerol compounds are present in the nutrient broth, probably in the meat extract which is one of its components. Only slightly higher activities were found in cells grown on nutrient broth plus glycerophosphate than in cells grown on nutrient broth alone. Therefore, it can be concluded that exogenous glycerophosphate cannot induce the synthesis of the glycerol-degrading enzymes in P. aeruginosa.

The ability of glucose to cause repression of synthesis of glycerol-degrading enzymes was determined in two ways. Extracts were prepared from cells grown in a mixture of glucose and glycerol and also from cells grown on glucose and exposed to a mixture of glucose and glycerol for 2.5 hours. In both cases, when the cells were harvested for the preparation of the extract, the supernatant fluid was analyzed for glucose

to be certain that it had not been exhausted from the medium. Both the kinase and dehydrogenase activities in the two extracts were considerably higher than in glucose-grown cells. It is apparent from these data that synthesis of glycerol-degrading enzymes is not subject to catabolite repression in P. aeruginosa.

B. Isolation of Glycerol Mutants

The fact that exogenously supplied glycerophosphate did not act as an inducer of glycerol-degrading enzymes in P. aeruginosa could be due to lack of a transport system for glycerophosphate. In order to determine whether endogenously formed glycerophosphate might be the true inducer of the enzymes, it was desirable to isolate a mutant unable to form glycerophosphate, i.e., a mutant lacking glycerol kinase.

Glycerol-negative mutants previously isolated in this laboratory had been of two types. Those which had a single defect, i.e., those which grew normally on glucose minimal medium, but did not grow on glycerol, all had very high reversion rates. Those which did not revert were also methionine auxotrophs and resistant to phage infection. The mutant selections used in the present study were designed to yield mutants which had no auxotrophic requirements since all isolation procedures included growth on glucose minimal medium as one step in the selection of mutants. Mutants selected by mutagenesis followed by antibiotic enrichment were expected to have no metabolic defect other than that in glycerol metabolism. Other mutants were selected by testing phage-resistant colonies, since Cowen (1968) and Mose (1970) had previously shown an apparent close linkage between inability to use glycerol and resistance to phage.

1. Nitrosoguanidine and Antibiotics

Cells of strain PA-1 (wild type), treated with nitrosoguanidine and antibiotics as described previously, were replicated from glucose minimal agar plates to glucose and glycerol minimal agar plates. Eight mutants which grew on glucose, but not on glycerol, were selected. They were numbered PA-1-801 to 803 from the procedure using carbenicillin and PA-1-809 to 813 from the D-cycloserine enrichment.

2. Phage-resistant Mutants

Each phage-resistant colony from plates containing Pø-6 phage was transferred with an inoculating needle to nutrient agar, glucose minimal agar and glycerol minimal agar plates. Colonies which grew on glucose, but not on glycerol, were selected and tested in liquid medium containing glucose or glycerol. Five mutants were obtained by this method. They were numbered PA-1-804 to 808. Another mutant, PA-1-800, was obtained by picking phage-resistant colonies from a Pø-6 phage plate prepared by R. Green.

C. Characterization of Glycerol Mutants

1. Growth Studies

Since glycerol-negative mutants of PA-1 isolated by Cowen (1968) had been shown by Mose (1970) to be unable to grow on gluconate, histidine or mannitol, the mutants isolated in the present study were tested for growth on these carbon sources as well as on glycerol. Several mutants isolated in previous studies were also included in this experiment, both for comparison with the newly-isolated mutants and as a check on their stability, since it was planned to use some of the mutants for enzyme studies. Mutants included were: PA-1-55 and PA-1-64, isolated

by Cowley as methionine auxotrophs; PA-1-92 through PA-1-97, isolated by Green (1969) as methionine auxotrophs; and PA-1-623 and PA-1-652, isolated by Cowen (1968) as glycerol-negative mutants.

Methionine was added to all media at a concentration of 17 μg/ml. Growth was measured as optical density at 540 nm. Readings were made at intervals of 2 hr for the first 12 hr of aeration and a final reading was made after 24 hr. The 24-hr readings are given in Table II. Mutants PA-1-92 through PA-1-97, PA-1-623 and PA-1-652 did not grow on any of the carbon sources tested. Mutants PA-1-55 and PA-1-64 and mutants of the 800 series grew on all the carbon sources with the single exception that PA-1-64 did not grow on histidine. The rates of growth and the total amounts of growth for these mutants were very similar to wild type on gluconate and histidine. Growth on both glycerol and mannitol is somewhat difficult to interpret since the wild type grows relatively poorly on both of these compounds. Growth of the mutants was better than that of the wild type in some cases, and poorer in others on both glycerol and mannitol.

Growth on glycerol of mutants of the 800 series, which had been isolated as glycerol-negative mutants on agar medium, could be due to formation of glycerol-degrading enzymes with reduced levels of activity (i.e., "leakiness") or to the presence of wild type revertants. In an effort to distinguish between these two possibilities, the wild type and each of the 800 series mutants were transferred to fresh glycerol minimal medium. A small inoculum, 0.05 to 0.1 ml, was transferred from each of the cultures shown in Table II, and the tubes were incubated with shaking at 37 C for approximately 46 hr. Optical density readings were made at intervals. Final optical densities are shown in Table III.

TABLE II

GROWTH OF WILD TYPE AND MUTANTS ON DIFFERENT CARBON SOURCES PLUS METHIONINE

Organism	Growth Media			
	Glycerol	Gluconate	Histidine	Mannitol
PA-1	0.459	0.751	0.803	0.330
PA-1-55	0.403	0.716	0.878	0.218
PA-1-64	0.491	0.776	0.058	0.523
PA-1-92	0.081	0.078	0.084	0.084
PA-1-93	0.048	0.054	0.068	0.052
PA-1-94	0.100	0.081	0.089	0.082
PA-1-95	0.084	0.080	0.101	0.084
PA-1-96	0.068	0.059	0.074	0.062
PA-1-97	0.051	0.047	0.053	0.042
PA-1-623	0.057	0.048	0.062	0.053
PA-1-652	0.064	0.069	0.085	0.067
PA-1-800	0.211	0.782	0.810	0.643
PA-1-801	0.260	0.776	0.733	0.274
PA-1-802	0.406	0.673	0.757	0.417
PA-1-806	0.342	0.727	0.862	0.323
PA-1-807	0.894	0.757	0.878	0.516
PA-1-808	0.462	0.757	0.727	0.789
PA-1-809	0.301	0.757	0.903	0.290
PA-1-810	0.472	0.776	0.810	0.658
PA-1-811	0.414	0.770	0.824	0.658
PA-1-812	0.530	0.789	0.824	0.710
PA-1-813	0.498	0.776	0.810	0.862

Methionine was added to each growth medium.

All optical density readings reported were taken at 24 hr at 540 nm.

TABLE III

GROWTH ON GLYCEROL OF MUTANTS PREVIOUSLY GROWN ON VARIOUS CARBON SOURCES

Mutants	Optical Density				
	Experiment and hours growth*				
	1-24 hr	2-46.5 hr	3-46.5 hr	4-46.5 hr	5-46.5 hr
PA-1-800	0.211	0.817	0.475	0.721	0.332
PA-1-801	0.260	0.776	0.491	0.939	0.349
PA-1-802	0.406	0.155	0.447	0.979	0.648
PA-1-806	0.342	0.542	0.704	0.959	0.854
PA-1-807	0.894	0.824	0.292	1.071	0.903
PA-1-808	0.462	0.930	0.598	0.615	0.886
PA-1-809	0.301	0.010	0.447	0.894	0.688
PA-1-810	0.472	0.653	0.063	0.264	0.036
PA-1-811	0.414	0.312	0.292	0.390	0.340
PA-1-812	0.530	0.233	0.066	0.272	0.057
PA-1-813	0.498	0.001	0.081	0.372	0.111
PA-1	0.459	0.757	0.545	0.930	0.390

Growth readings are expressed as optical density at 540 nm.

*Experiment 1 repeats the data from Table I for growth on glycerol plus methionine. Experiments 2 to 5 show final growth of 0.05 - 0.10 ml of cell suspension transferred to fresh glycerol minimal medium; they were previously grown on gluconate, histidine, mannitol and glycerol, respectively.

Again there was a great deal of variation in amounts of growth on glycerol. Since all of the strains were able to grow as well as the wild type in at least one tube, it is apparent that all mutants of the 800 series are capable of reverting to wild type. It is possible that PA-800, 801, 802, 806, 807, and 808 may also be leaky. Mutants PA-1-809, 810, 811, 812, and 813 may have lower reversion rates than do the others, since each of these failed to grow in at least one tube. It is interesting to note the varia bility in growth of the wild type. This variation is typical of the growth of P. aeruginosa on glycerol.

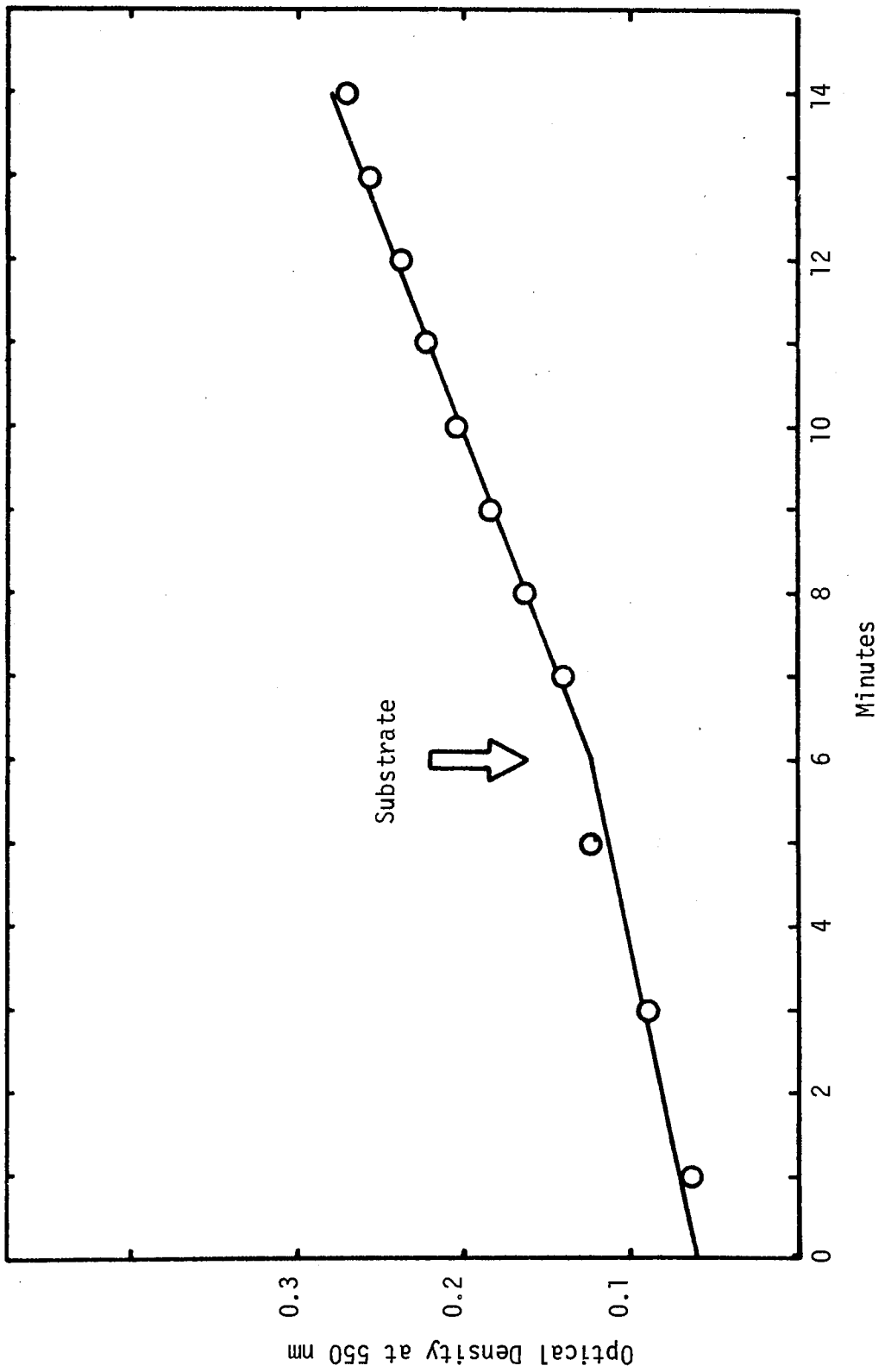
Growth on glycerol was checked repeatedly for mutants of the 800 series and was compared each time with growth of the wild type. Each mutant of the series differed sufficiently from the wild type in several experiments to justify the conclusion that these are defective in glycerol metabolism although their reversion rates are too high to allow clear-cut differentiation from the wild type in a single experiment.

2. Assay of L-α-Glycerophosphate Dehydrogenase in Whole Cells

The method devised by Cowen (1968) to test for L-α-glycerophosphate dehydrogenase activity in whole cells was used as a preliminary screening procedure with 19 mutants. Mutants screened by this method included the following: PA-1-92, 93, 94, 95, 96, 97, 623, 652, 800, 801, 802, 806, 807, 808, 809, 810, 811, 812, and 813. Many of the mutants had extremely high endogenous activity, which made results with the whole cell assay difficult to interpret; therefore, negative results were discounted as unreliable. Those which appeared to lack dehydrogenase activity by this method were: PA-1-92, 93, 94, 95, 96, 623, 800, 801, 806, 809, 810, and 812. Those which were definitely positive included PA-1-807, 808, 811, and 813. Figure 2 shows dehydrogenase activity as

Figure 2. Measurement of L-α-Glycerophosphate Dehydrogenase Activity in Whole Cells of Pseudomonas aeruginosa, PA-1.

PA-1 cells were prepared as described in the text by growth on 0.5 per cent glucose minimal medium, induction of one-half the cells in 1.0 per cent glycerol medium, washing and storage in ice-cold distilled water to remove endogenous substrates, and freezing to increase permeability. Dehydrogenase activity was measured as reduction of MTT before and after addition of the substrate, α-glycerophosphate. The non-induced portion of the culture, similarly treated, served as a control. The optical density for the induced cells was subtracted from that of the uninduced cells to yield a net measurement.


Optical Density at 550 nm
0.1
0.2
0.3
0
2
4
6
8
10
12
14
Minutes
Substrate

measured in whole cells of the wild type. Figure 3 shows activity measured in two mutants. One of these, PA-1-813 was positive, i.e., it had dehydrogenase activity, and the other, PA-1-92, was negative, i.e., it had no activity. These results as shown in the next section, were confirmed by assays with cell-free extracts. However, other mutants which did not appear positive in the whole cell assay did have dehydrogenase activity which was measurable in extracts.

3. Enzyme Activities in Cell-free Extracts

a. Non-reverting Mutants

Cowen (1968) had previously shown that mutant PA-1-663 lacked L-α-glycerophosphate dehydrogenase activity and had approximately 30 per cent of the triose phosphate isomerase activity of the wild type. Since activities of the glycerol enzymes had not previously been determined in mutants of the PA-1-92 series isolated by Green (1969), an extract was prepared from glycerol-induced cells of strain PA-1-93. Extracts were also prepared from two of Cowen's mutants, PA-1-663, which had been previously assayed, and PA-1-623, which had not been previously studied. Data for these mutants are shown in Table IV. Data for the wild type are shown to allow comparison of activities.

All of these mutants have essentially no dehydrogenase activity. Isomerase activity was measured in all three mutants, and in these mutants the enzyme was present at a very low level, approximately 5 per cent of that in the wild type. All three mutants had elevated kinase activity as compared with the wild type.

b. Reverting Mutants

Extracts were prepared from 7 of the mutants of the 800 series.

Figure 3. Measurement of L-α-Glycerophosphate Dehydrogenase Activity in Whole Cells of Glycerol Mutants.

The experiment was performed as described in Figure 2 using mutant cells rather than wild type cells.

Mutant PA-1-813 (o); PA-1-92 (Δ).

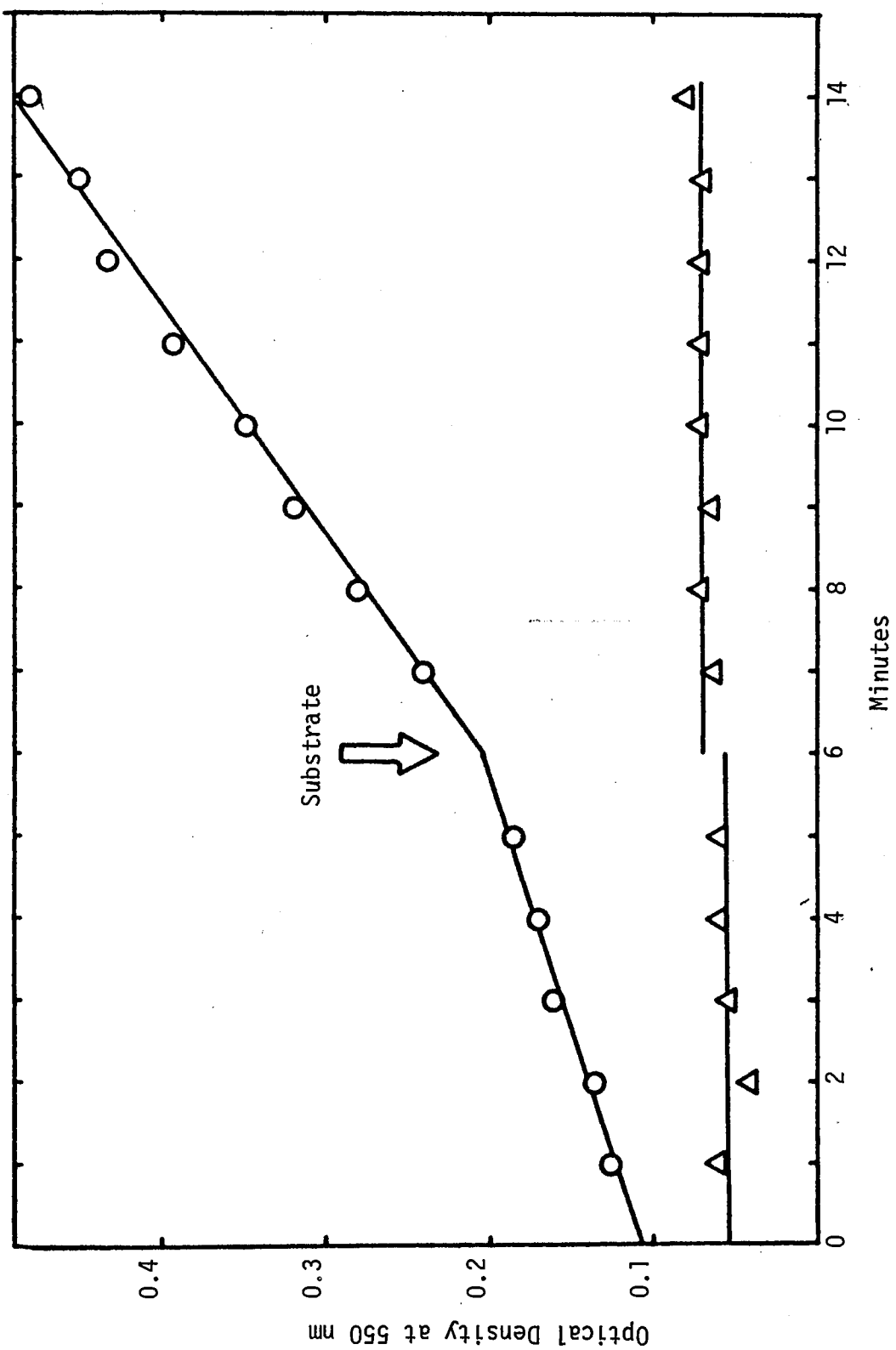

Optical Density at 550 nm
0.1
0.2
0.3
0.4
Substrate
0
2
4
6
8
10
12
14
Minutes

TABLE IV

SPECIFIC ACTIVITIES OF GLYCEROL ENZYMES IN CELL-FREE EXTRACTS OF NON-REVERTING MUTANTS

Strain	Specific Activities (n moles/min/mg protein)		
	Kinase	Dehydrogenase	Isomerase
PA-1	5.55	5.69	393
PA-1-93	12.83	0.08	21.6
PA-1-623	14.50*	0.17*	17.7
PA-1-663	17.24	0	17.9

*The data for these assays are the averages for two different cell extracts.

Those which appeared to have the highest reversion rates were not used for enzyme studies. All cultures were grown on glucose minimal medium and induced by aeration in glycerol minimal medium for 4 hr. Enzyme activities measured in these extracts are shown in Table V. Dehydrogenase activities in 6 of the strains were 40 per cent or more of the wild type and are possibly not significantly different from wild type. Kinase activities in strains 809, 811, and 813 were 60 per cent or more of the wild type activity. Mutant PA-1-810 had an elevated kinase activity comparable to that found in the non-reverting dehydrogenaseless mutants.

As stated previously, one of the objects of the mutant isolations included in this investigation was to obtain a kinaseless mutant. The first set of data shown for strain PA-1-812 indicates that this strain lacks kinase activity. Dehydrogenase activity in this extract was also very low. When a second extract was prepared from strain 812, the kinase activity was similar to the wild type activity and the dehydrogenase activity was approximately 6 times that found in the first extract. It was mentioned previously that this mutant, PA-1-812, may have reverted to some extent during transfer. The enzyme activities of both glycerol kinase and glycerophosphate dehydrogenase apparently may be recovered by reversion.

4. Phage Sensitivity of Glycerol Mutants

Since some previously isolated glycerol mutants had been found to be resistant to phage, mutants of the 800 series, both those originally selected for phage resistance and those selected as glycerol negative only, were tested for sensitivity to phage F116. This information was needed if transduction studies were to be done, and it was also of

TABLE V

SPECIFIC ACTIVITIES OF GLYCEROL ENZYMES IN CELL-FREE EXTRACTS OF REVERTING MUTANTS

Strain	Specific Activities (n moles/min/mg protein)	
	Kinase	Dehydrogenase
PA-1	5.55	5.69
PA-1-800		2.60
PA-1-801		4.63
PA-1-809	3.55	2.52
PA-1-810	11.86	2.34
PA-1-811	3.27	5.48
PA-1-812*	0	1.12
PA-1-812*	5.45	6.53
PA-1-813	4.07	4.22

*Two cell-free extracts of PA-1-812 were prepared at different times.

interest to determine whether the two mutations frequently occur simultaneously.

The phage suspension was diluted to a concentration of 10^4 PFU/ml and 0.1 ml was plated in an agar overlay with one drop of each mutant culture on separate plates. Mutants previously shown by Mose (1970) to be phage-resistant were included as controls and as a check on their stability. The wild type parent was also included as a control. The results of this experiment are shown in Table VI.

Mutants PA-1-92 through 97, PA-1-623 and PA-1-652 were still completely resistant to phage infection as previously reported. Mutants 801, 806, 807, and 808, isolated in the present study, were also completely resistant. The other mutants of the 800 series were partially sensitive, i.e., all produced turbid plaques which may be indicative of a mixture of sensitive and resistant cells. This is consistent with the growth data in Tables II and III which showed frequent reversion to wild type in these mutants, and is also consistent with the enzyme data in Table V. Alternatively, the turbid plaques obtained with the glycerol mutants could indicate lowered phage yield due to a requirement for the glycerol enzymes in some step of phage production. To determine which of these explanations is correct, mutants 807, 809, 811, and 812 were grown through several transfers on glycerol minimal medium to select for revertants. These cultures were again plated with phage Pø-6. All produced plaques similar in morphology to those produced on wild type cells. Therefore, it appears that the ability to grow on glycerol and sensitivity to phage are lost and regained simultaneously by mutation and reversion.

TABLE VI

PHAGE SENSITIVITY OF GLYCEROL MUTANTS

Completely Resistant Strains	Sensitive Strains	Plaque Morphology
801	PA-1 (wild type)	Clear
806	800	Very turbid
807	802	Turbid
808	809	Turbid
92	810	Turbid
93	811	Tiny, turbid
94	812	Very turbid
96	813	Tiny, turbid
97		
623		
652		

D. Transduction Studies

One of the objectives of the mutant isolation in the present study, as previously stated, was to obtain glycerol-negative mutants which were phage-sensitive and capable of growth on minimal medium, and which would be sufficiently stable to allow study of the linkage between the methionine and glycerol loci by transduction. Growth studies, enzyme assays and tests of phage sensitivity indicated that the mutants obtained were not ideally suited for use in transduction, but three mutants which appeared to have lower reversion rates were used in attempts to co-transduce the glycerol defect and methionine sufficiency into three methionine auxotrophs which had no defect in glycerol metabolism.

The methionine mutants PA-1-27, PA-1-55, and PA-1-64 were resistant to most phages for P. aeruginosa, but Mose (1970) had found that they were sensitive to phage P-ø-5. Mutants PA-1-811, 812, and 813 were also found to be sensitive to this phage and plate stocks of these mutants were prepared. Titers of the plate stocks were approximately 10^{10} PFU per ml.

Transductions were carried out by the method described by Bruce (1965) in which approximately 10^9 recipient cells were spread on the surface of an agar medium selective for transductants and drops of plate stock were placed on the surface at marked points. The background growth serves as a control for reversion. In this experiment it was necessary to select only for transfer of the wild type methionine marker and to pick off and test each presumed transductant colony individually for the non-selected marker. Accordingly, PA-1-27, 55, and 64 were spread separately on glucose minimal agar plates and phage suspensions grown on PA-1-811, 812, and 813 were spotted on each. There was a

large amount of background growth, consistent with the usual reversion found with these cultures. However, colonies from the areas treated with phage were picked off individually with an inoculating needle and transferred to glucose minimal agar. After colonies developed, they were transferred to plates of glycerol minimal medium, glucose minimal medium and medium containing glycerol plus methionine. Several colonies which grew poorly on glycerol plus methionine were tested in liquid medium to determine whether the attempted transduction had altered the characteristics of the cells as expected. Each culture was inoculated into glucose minimal medium, glucose plus methionine and glycerol plus methionine. Growth was measured at intervals during aeration, and aeration was continued for approximately 50 hr. At the end of this time, fairly good growth had occurred in all tubes. However, considerable differences in growth were evident at 24 hr for several of the cultures. These data are shown in Table VII. The wild type, PA-1, was included in this experiment but growth data for the mutants are taken from other experiments and are included in the table for comparison. Since all of the methionine auxotrophs used in this study are capable of reverting, it is not possible to say with any degree of certainty that the ability to grow in the absence of methionine is a result of transduction rather than reversion. The defect in glycerol metabolism apparently can be transferred by transduction, however, since three of the cultures show this characteristic. As an unselected marker, its presence cannot be explained otherwise. In only one case was co-transduction of the two markers possibly achieved. Further study of the linkage of these markers will require non-reverting mutants for both markers.

TABLE VII

GROWTH STUDIES OF POSSIBLE TRANSDUCTANTS

Cells	Glucose Minimal	Glucose-Methionine	Glycerol-Methionine	Marker Transferred
PA-1	0.838	0.870	0.392	
27(811)*	0.018	0.803	0.305	
27(812)	0.199	0.733	0.140	Gly^-
27(813)	0.014	0.624	0.029	Gly^-
55(811)	0.803	0.886	0.093	$Gly^-_+Meth^+$
55(812)	0.824	0.854	0.256	$Meth^+_+$
55(813)	0.838	0.810	0.238	$Meth^+_+$
64(811)	0.912	0.782	0.485	$Meth^+_+$
64(812)	0.803	0.456	0.602	$Meth^+$
64(813)	0.475	0.423	0.372	
PA-1-27	0.074		0.417	
PA-1-55	0.027		0.403	
PA-1-64	0.028		0.491	

*The number in parentheses is that of the donor cell, i.e., the mutant on which the phage used for transduction had been grown. For example, cells designated as 27(811) are those selected as possible transductants, by the procedures described in the text, from a plate containing cells of PA-1-27 treated with phage grown on PA-1-811.

E. Transport of Glycerol

1. Measurement by Osmotic Effect

The method used by Sanno, et al. (1968) to detect facilitated diffusion of glycerol in *E. coli* was employed with *P. aeruginosa* to determine whether this organism takes up glycerol by the same mechanism. The method was also used to study control of the uptake system and the effect of inhibitors. Parallel measurements were made in several experiments with *E. coli* (strain 45, OSU Microbiology Department) to allow comparison of the two organisms with respect to glycerol uptake.

Initially, the effect of additions of 2.4 M NaCl and distilled water on both organisms was determined. Figure 4a shows that the addition of 0.5 ml of 2.4 M NaCl to 2.5 ml of cell suspension caused an increase in optical density. This is due to cytoplasmic shrinkage because the cell is only slightly permeable to NaCl. Figure 4b shows the effect of simple dilution of a cell suspension with 0.5 ml of distilled water. Equilibration in this case is very rapid. The changes in optical density recorded for these two treatments are similar to those reported by Sanno, et al. for *E. coli* K12.

a. Control of Glycerol Transport

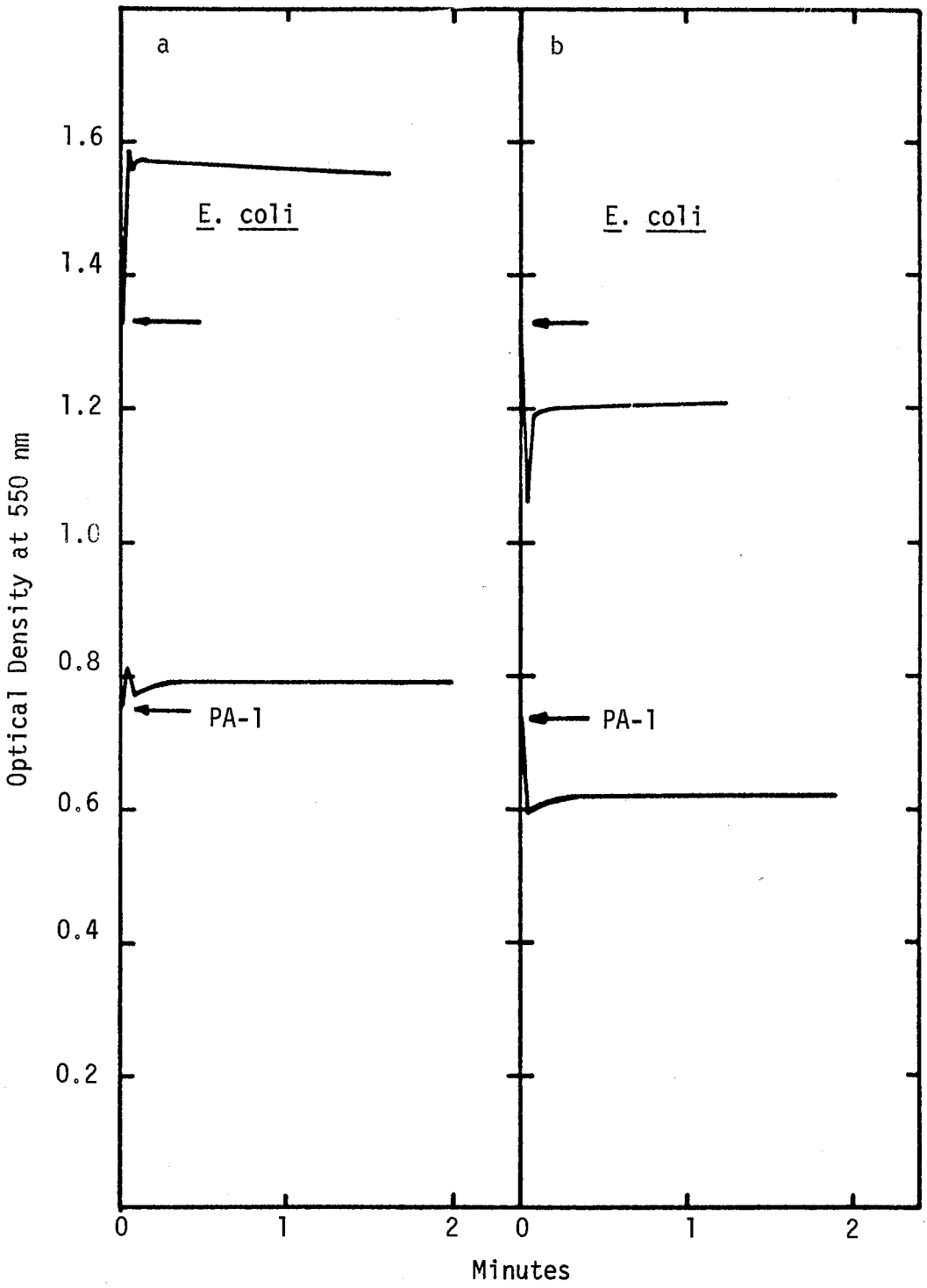
Sanno, et al. (1968) showed that facilitated diffusion of glycerol in *E. coli* was an inducible process and that it was constitutive in a mutant constitutive for metabolism of glycerol. They also showed that L-α-glycerophosphate was the true inducer for the facilitated diffusion system since no induction occurred in a kinaseless mutant exposed to glycerol.

The effect of induction with glycerol on glycerol uptake was

Figure 4. Measurement by Osmotic Effect of the Cellular Permeability of *P. aeruginosa* and *E. coli* to Sodium Chloride and Water.

Cells were prepared as described in the text by growth on nutrient broth, washing with 0.02 M, pH 7.5, Tris buffer, and resuspending in 4 nM, pH 7.5, Tris buffer. 2.5 ml of this cell suspension were placed in a cuvette and 0.5 ml of 2.4 M NaCl or water was introduced into the cuvette with a hypodermic syringe. The change in optical density was recorded within 5 sec using a Cary 14 Spectrophotometer.

(a) Addition of NaCl; (b) Addition of water.


a
b
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
E. coli
E. coli
PA-1
PA-1
Optical Density at 550 nm
0
1
2
0
1
2
Minutes

determined for both E. coli and P. aeruginosa. These data are shown in Figure 5. Recordings obtained with E. coli were similar to those reported by Sanno, et al. Figures 5a and 5b show the change in optical density produced by addition of 0.5 ml of 4.8 M glycerol to suspensions of uninduced E. coli and P. aeruginosa, respectively. Equilibration was relatively slow due to lack of the inducible facilitated diffusion system. Figures 5c and 5d show the rapid equilibration which occurs when hypertonic glycerol is added to cells previously induced with glycerol. This experiment demonstrates that P. aeruginosa, like E. coli, possesses an inducible mechanism for rapid uptake of glycerol.

Sanno, et al. did not determine whether the uptake system for glycerol was subject to catabolite repression nor did they attempt to demonstrate an energy requirement by studying the effect of metabolic inhibitors. Both of these questions were investigated in the present study.

Figure 6 shows the changes in optical density recorded after addition of hypertonic glycerol to E. coli and P. aeruginosa grown in a mixture of glucose and glycerol. While the formation of the uptake system for glycerol in E. coli is completely repressed by glucose, there is no apparent effect of glucose in P. aeruginosa.

Since a large number of uptake experiments using this method were carried out, the remainder of the data is presented in tabular form. A plus sign is used in the tables to indicate a curve similar to those shown in Figures 5c and 5d, i.e., rapid equilibration due to the presence of the inducible uptake system. A minus sign is used to indicate slow equilibration due to simple diffusion, i.e., a curve showing no facilitated diffusion and similar to those in Figures 5a and 5b.

Figure 5. Measurement by Osmotic Effect of the Permeability to Glycerol of *P. aeruginosa* and *E. coli*, Uninduced and Induced.

The cells were prepared as described in Figure 3 except that they were grown in 1.0 per cent glucose minimal medium instead of nutrient broth for uninduced cells. For induction, cells were grown in 1.0 per cent glycerol minimal medium. Glycerol, at a concentration of 4.8 M, was added rapidly from a syringe.

(a) *E. coli*, uninduced; (b) *P. aeruginosa*, uninduced; (c) *E. coli*, induced; (d) *P. aeruginosa*, induced.

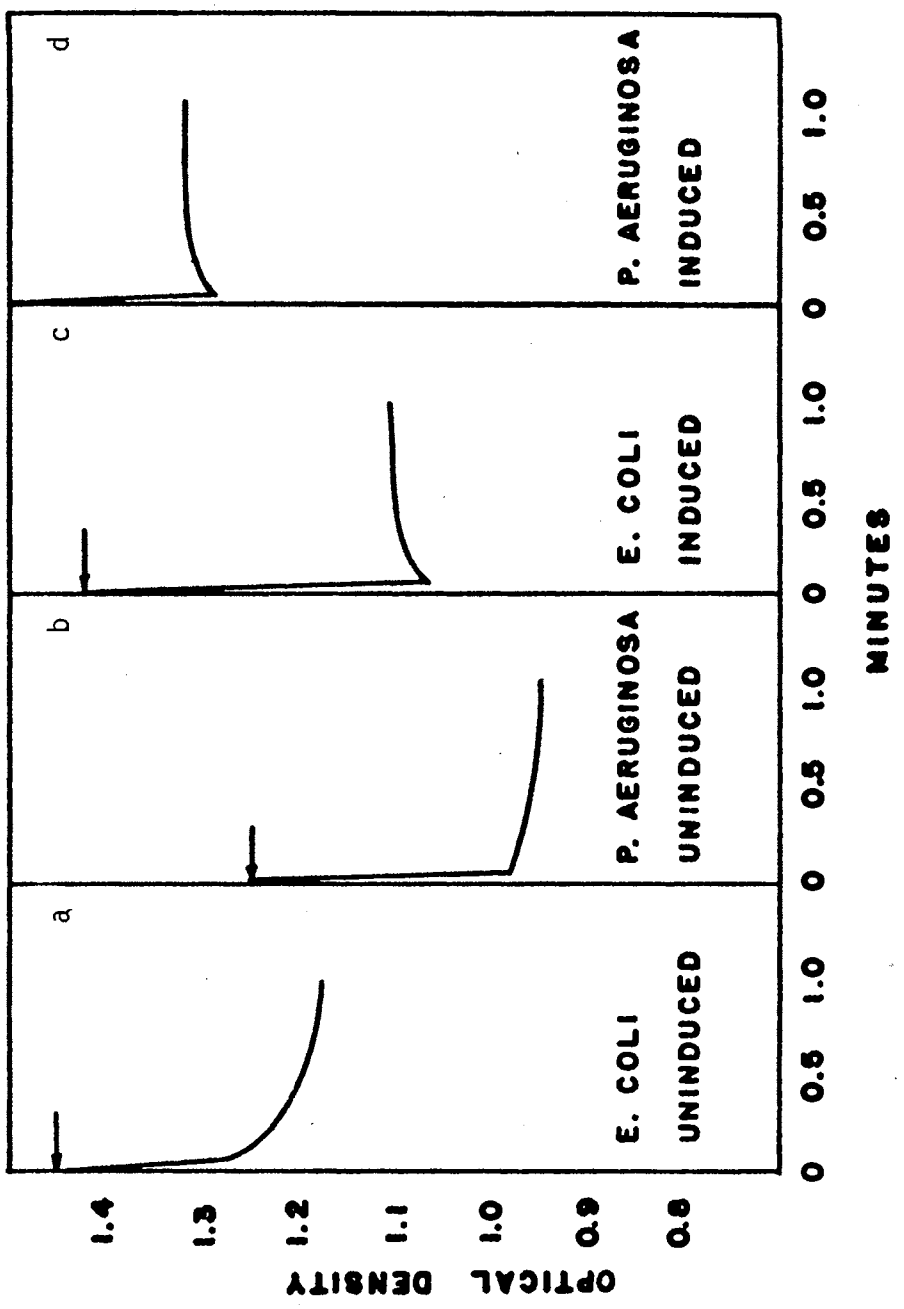

a
b
c
d
E. COLI UNINDUCED
P. AERUGINOSA UNINDUCED
E. COLI INDUCED
P. AERUGINOSA INDUCED
OPTICAL DENSITY
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0
0.5
1.0
MINUTES

Figure 6. Measurement by Osmotic Effect of the Permeability to Glycerol of *P. aeruginosa* and *E. coli* Grown on Glucose Plus Glycerol.

The cells were prepared as described in Figure 5 except that they were grown in minimal medium containing 0.5 per cent glycerol and 0.5 per cent glucose.

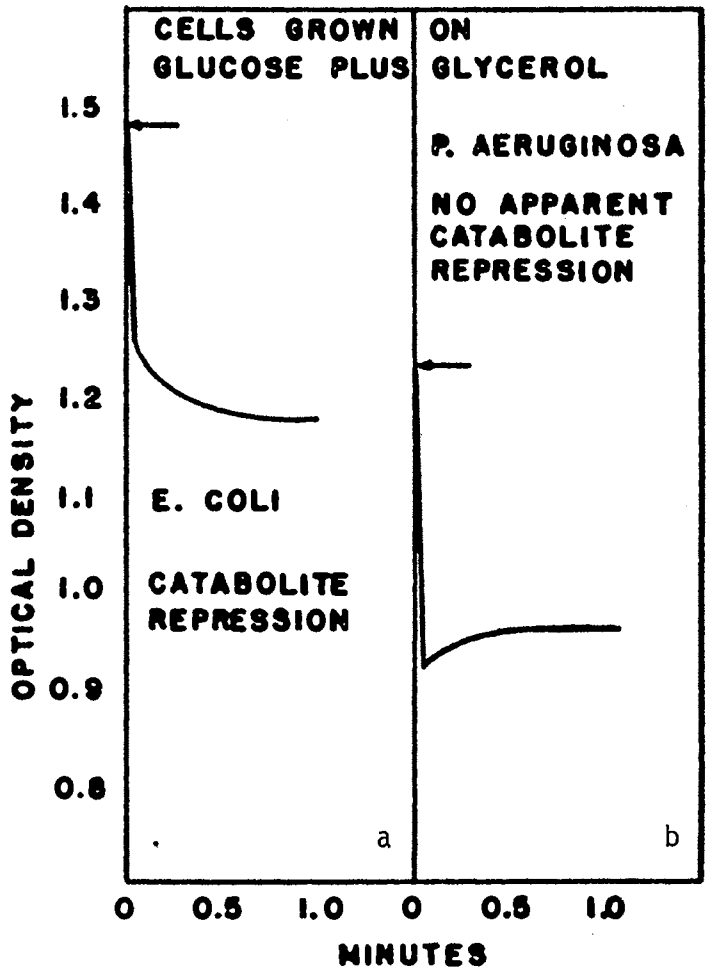

CELLS GROWN ON GLUCOSE PLUS GLYCEROL
P. AERUGINOSA
NO APPARENT CATABOLITE REPRESSION
E. COLI
CATABOLITE REPRESSION
OPTICAL DENSITY
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
a
b
0
0.5
1.0
0
0.5
1.0
MINUTES

Table VIII shows data for transport of glycerol in three organisms, E. coli strain 45, Achromobacter sp. and P. aeruginosa strain PA-1. The Achromobacter sp. was included since previous studies of the effect of glucose on glycerol metabolism had been carried out with this organism as well as with E. coli strain 45 (Tsay, 1968). Cells were grown or induced with different compounds to determine the effect of induction on uptake.

The uptake studies using glycerol with P. aeruginosa agree with the the enzyme data shown in Table I except for cells grown in medium containing glycerophosphate. No rapid uptake was observed for cells grown on sodium lactate or glucose in minimal medium or on 1.0 per cent casamino acids. However, cells grown on nutrient broth, glycerol, or casamino acids containing glycerophosphate did exhibit rapid uptake. Catabolite repression was again checked under two different conditions. Cells were grown on glucose plus glycerol or were grown on glucose and then exposed to a mixture of glucose and glycerol for 2.5 hr. Glucose was still present in the medium when the cells were harvested, as shown by the Glucostat assay. Again, no catabolite repression was found. It is interesting that glycerophosphate apparently is capable of acting as the inducer for the facilitated diffusion system for glycerol even though no induction of the catabolic enzymes could be demonstrated in P. aeruginosa.

In parallel experiments using E. coli and Achromobacter sp., it was shown that these two organisms were alike in the control of formation of the glycerol uptake system. In both organisms, facilitated diffusion was present in cells grown on glycerol or glycerophosphate. Both organisms are able to use glycerophosphate as the sole substrate

TABLE VIII

INDUCTION OF SYSTEM FOR GLYCEROL UPTAKE IN E. COLI 45, ACHROMOBACTER AND P. AERUGINOSA

Growth Medium	Cells		
	E. coli 45	Achromobacter	PA-1
Casamino acids	-		-
Nutrient broth	+		+
Glycerol	+	+	+
Glucose	-	-	-
Glycerophosphate*	+	+	+
Glucose plus** glycerol	-	-	+
Lactate			-
Glucose grown, induced with glycerol + glucose			+

*One per cent casamino acid was added to the growth medium for PA-1.

**Glucose was still present in the medium when the cells were harvested.

in minimal medium, unlike P. aeruginosa, which cannot use this compound for growth. In both organisms, growth of the cells on glucose plus glycerol failed to induce the glycerol uptake system; i.e., in both it was subject to catabolite repression.

b. Energy Requirement for Glycerol Transport

To determine whether rapid uptake of glycerol in P. aeruginosa is due to facilitated diffusion or to an energy-requiring active transport system, the effect of three different inhibitors was studied using the same technique for detecting rapid uptake. In some experiments, the inhibitors were added just prior to addition of substrate, and in others the cells were pre-incubated with inhibitor for at least 30 minutes. Three different types of cell suspensions were checked with each inhibitor. These were cells grown in nutrient broth, cells grown in glucose minimal medium, and cells grown in glucose minimal medium, then induced with glycerol.

Data for these experiments are shown in Table IX. Cells grown on nutrient broth or cells induced with glycerol after growth on glucose exhibited rapid uptake of glycerol and no effect on uptake was found when cells were treated with KCN, 2,4-dinitrophenol or sodium azide. All of these inhibitors are effective in preventing energy-coupled active transport. Therefore, it can be concluded that transport of glycerol in P. aeruginosa is not an energy-requiring process and is due to facilitated diffusion. Glucose-grown cells, included in many of the experiments as controls, never exhibited rapid uptake of glycerol.

c. Glycerol Transport in Mutants

Several of the mutant strains used in other portions of this study

TABLE IX

EFFECT OF INHIBITORS ON TRANSPORT OF GLYCEROL IN PSEUDOMONAS AERUGINOSA

Preparation of Cells*	Inhibitor Added (ml)				
	0.2 M KCN	15 mM DNP	0.3 M NaN_3	Pre-incubation (minutes)	Uptake
Nutrient Broth					+
Glucose					-
Glycerol					+
Nutrient Broth	0.1			37	+
Glucose	0.1			34	-
Glycerol	0.1			32	+
Glycerol	0.1			0	+
Glycerol	0.2			0	+
Glycerol	0.3			0	+
Nutrient Broth		0.1		0	+
Glucose		0.1		0	-
Glycerol		0.1		0	+
Glycerol		0.2		0	+
Glycerol		0.3		0	+
Nutrient Broth			0.1	37	+
Nutrient Broth			0.2	43	+
Nutrient Broth			0.3	37	+
Glucose			0.1	30	-
Glucose			0.2	39	-
Glucose			0.3	34	-
Glycerol			0.1	30	+
Glycerol			0.2	35	+
Glycerol			0.3	32	+
Glycerol			0.1	0	+
Glycerol			0.2	0	+
Glycerol			0.3	0	+

*Nutrient broth and glucose minimal medium were used for growth of cells. Glycerol was used for induction of cells grown on glucose.

The total volume was 3.0 ml. Pre-incubation time is minutes cells were exposed to inhibitor before uptake was measured.

were examined for ability to take up glycerol by facilitated diffusion. Cells were grown on glucose minimal medium, with or without added methionine as required, and were induced with glycerol in most cases. Two of the mutants, PA-1-93 and PA-1-623, were tested under three conditions: uninduced, induced in glycerol minimal medium, and induced in glycerol medium with methionine. These data are shown in Table X.

The facilitated diffusion system for glycerol was completely absent in mutants 97 and 623, under all conditions tested. However, glycerol was taken up by facilitated diffusion in all of the other mutants tested, both those of the 800 series and the three methionine auxotrophs, PA-1-27, 55, and 64.

2. Measurement with ^{14}C-Glycerol

To confirm the conclusions based upon measurements of uptake using the method of Sanno, et al. (1968) by more conventional methods and to study the rate of uptake, ^{14}C-glycerol was used. Uptake studies were carried out as described in Chapter II and the data were calculated as n moles ^{14}C-glycerol taken up per mg dry weight of cells. Curve a in Figure 7 shows that glycerol was taken up rapidly by glycerol-induced, wild type cells, reaching a plateau at approximately 2.5 min. Curve b shows that energy is not required for transport. A cell suspension similar to that used to obtain Curve a was incubated for 30 min after addition of 2.5 ml of 10 mM 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, and was then used to measure uptake of ^{14}C-glycerol. The rate of uptake was slightly less, but if active transport were involved, complete inhibition of uptake would be expected. Therefore, these results agree with those obtained by the method of Sanno, et al. (1968).

TABLE X

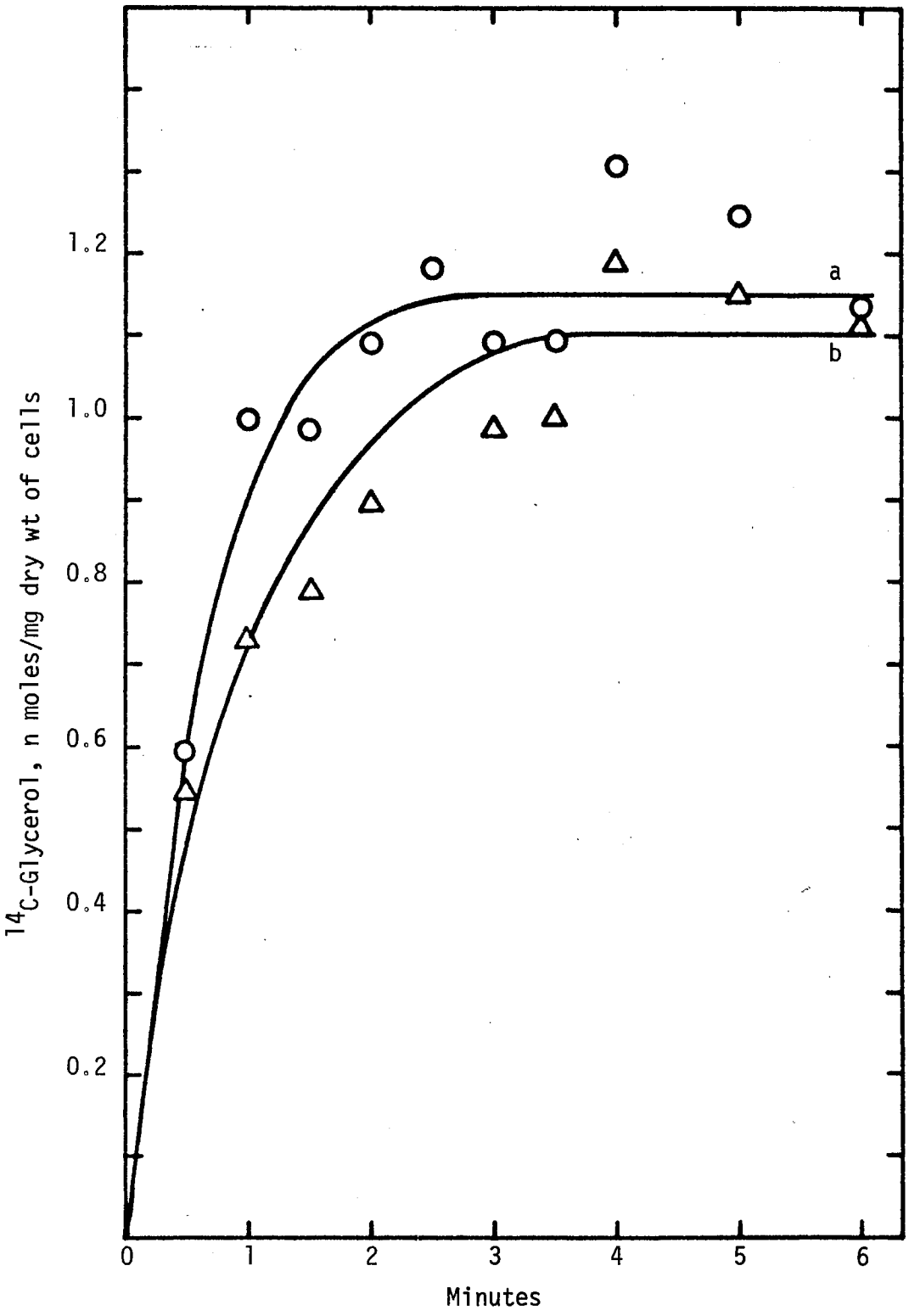
UPTAKE OF GLYCEROL BY GLYCEROL MUTANTS

Strain	Induced in	Uptake
PA-1	Glycerol	+
PA-1-27	Glycerol	+
PA-1-55	Glycerol	+
PA-1-64	Glycerol	+
PA-1-97	-	-
PA-1-623	-	-
PA-1-97	Glycerol	-
PA-1-623	Glycerol	-
PA-1-97	Gly. + Meth.	-
PA-1-623	Gly. + Meth.	-
PA-1-800	Glycerol	+
PA-1-801	Glycerol	+
PA-1-802	Glycerol	+
PA-1-809	Glycerol	+
PA-1-810	Glycerol	+
PA-1-811	Glycerol	+
PA-1-812	Glycerol	+
PA-1-813	Glycerol	+

Figure 7. Uptake of ^{14}C-Glycerol by P. aeruginosa With and Without the Addition of an Energy Uncoupler.

Cells were grown in 0.5 per cent glucose minimal medium, washed, induced in 0.5 per cent glycerol minimal medium, and diluted to 1.5 x 10^9 cells per ml. At zero time, 0.1 ml of ^{14}C-glycerol (5,068,070 dpm/ml) was added to 25 ml of cell suspension to a final concentration of 0.636 µM. One ml samples were withdrawn with a syringe and filtered through a Millipore filter (HA, 045 µ pore size) at 30 to 60 sec intervals. The filter pads were washed twice with 1 ml of ice-cold 0.01 per cent glucose minimal medium, placed in counting vials and dried with hot air. Scintillation fluid was added and the samples were counted in a liquid scintillation counter. A second experiment was done in the same way except that 2.5 ml of 10 mM 2.4 dinitrophenol was added to the cell suspension 30 min prior to the addition of ^{14}C-glycerol.

(a) PA-1 cells; (b) PA-1 with DNP.


1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
Minutes
^{14}C-Glycerol, n moles/mg dry wt of cells
a
b

Glucose-grown cells, not induced with glycerol, were used to measure the rate of uptake in the absence of the inducible system for facilitated diffusion. These data are shown in Figure 8, Curve a. Uptake of glycerol was much slower in uninduced cells. The level of radioactivity in the cells after 16 min was only 24 per cent of the plateau value reached in 2.5 min with induced cells.

One of the mutants which had been found to exhibit no facilitated diffusion, as measured by the osmotic effect, was also used in an experment with labeled glycerol. The data are shown in Figure 8, Curve b, for PA-1-623, induced with glycerol in minimal medium. There was a very slow uptake of glycerol which reached a plateau value less than 10 per cent of that found with the wild type. Approximately 10 min were required to reach the plateau. Apparently, a small amount of glycerol is able to enter the cell by simple diffusion, but this mutant seems to lack completely the inducible system for facilitated diffusion.

Kinetic constants for glycerol uptake were determined by measuring uptake with a series of concentrations of glycerol. Uptake was measured at each concentration by removing samples at 30-second intervals for 90 seconds. Each uptake curve was plotted and the initial rate was determined. In all cases, the 30-second sample was found to be appropriate for measurement of initial rate and these data were used in a reciprocal plot (Lineweaver and Burk, 1934) to determine the Km and Vmax for glycerol transport. The line of best fit was calculated using the linear regression method given by Steel and Torrie (1960). It was found that the kinetic data could best be fitted to two straight lines, one for the higher range of concentrations and another for lower concentrations. These plots are shown in Figure 9. For the lower

Figure 8. Uptake of ^{14}C-Glycerol in Wild Type Cells Grown on Glucose and in a Transport-negative Mutant, PA-1-623.

The experiment was carried out under conditions described in Figure 7, except that the induction step was omitted for PA-1 cells.

PA-1, glucose-grown (o); PA-1-623 (Δ).

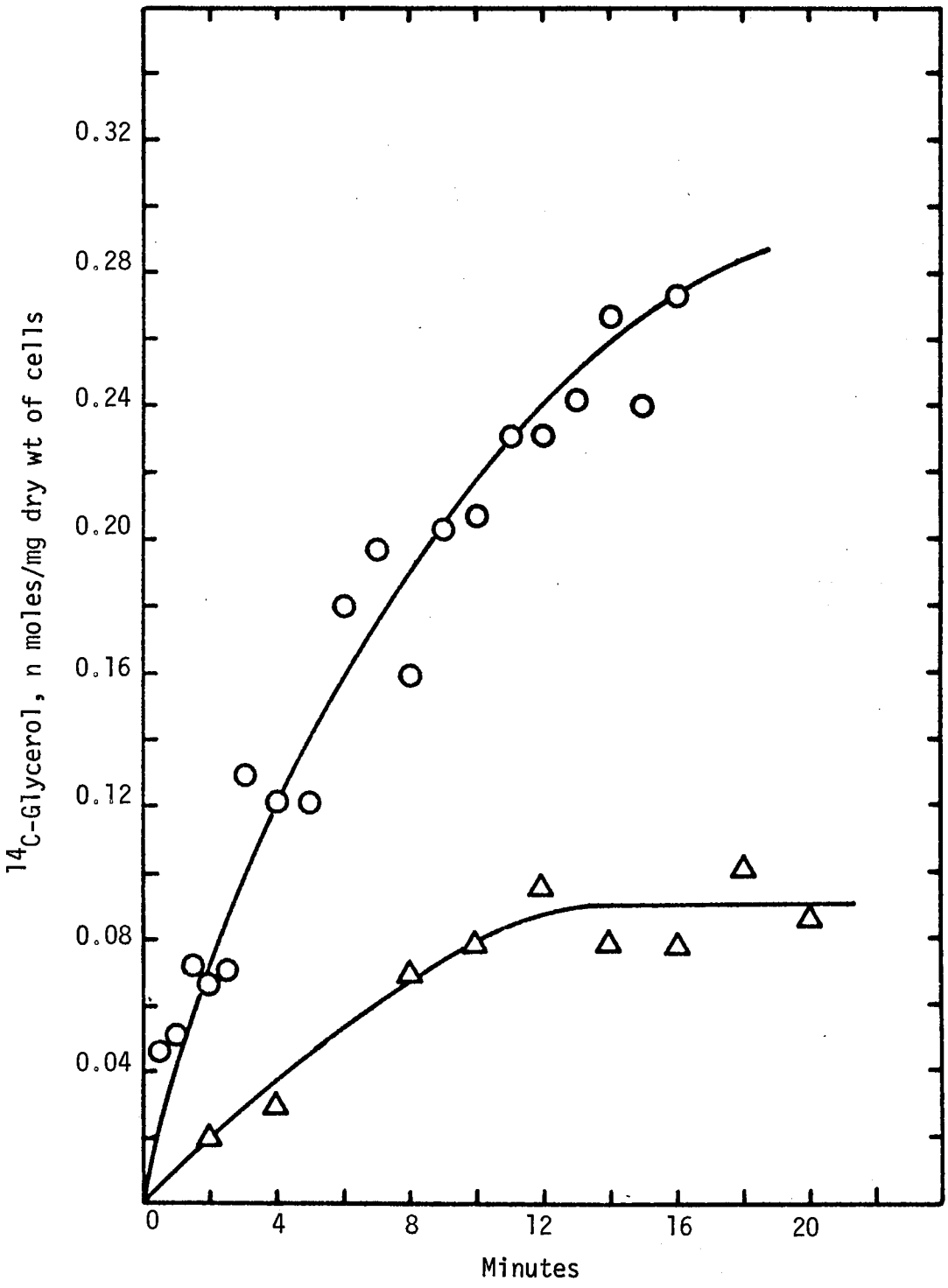
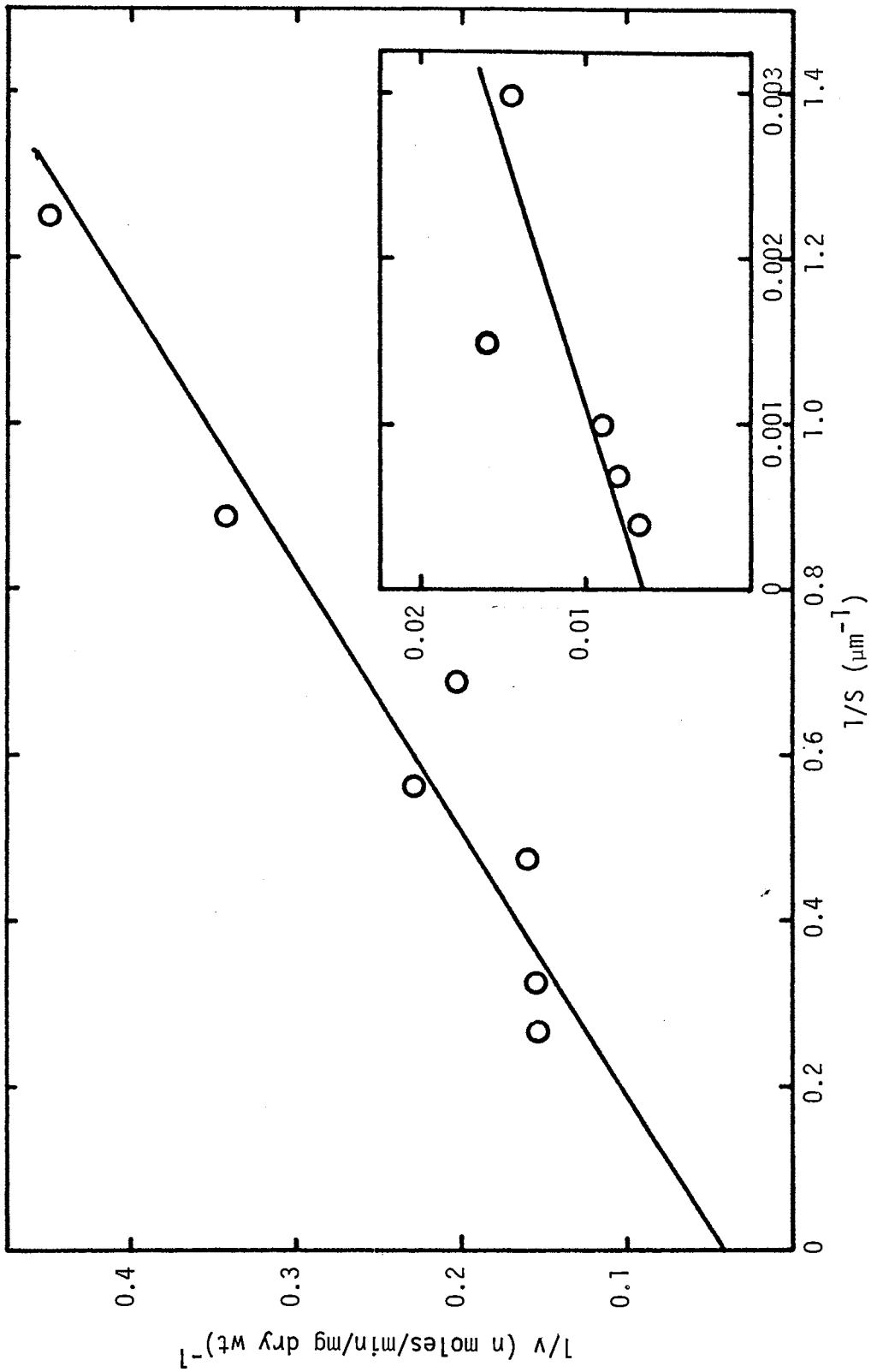

0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
^{14}C-Glycerol, n moles/mg dry wt of cells
0
4
8
12
16
20
Minutes

Figure 9. Double Reciprocal Plots of ^{14}C-Glycerol Uptake by *P. aeruginosa*.

Experiments using different concentrations of glycerol were carried out under the conditions described in Figure 7. Uptake was measured by removing samples at 30-second intervals for 90 seconds. The thirty-second uptake was used as a measure of initial rate for plotting.

Inset: Double reciprocal plot at high glycerol concentrations.


1/v (n moles/min/mg dry wt)$^{-1}$
0.4
0.3
0.2
0.1
0
0.2
0.4
0.6
0.8
1/S (μm^{-1})
0.02
0.01
0
0.001
1.0
0.002
1.2
0.003
1.4

concentrations, the Km was 7.8×10^{-6} M, and Vmax was 25 n moles per min per mg dry weight of cells. For higher concentrations, Km was 4.8×10^{-4} M, and Vmax was 151 n moles/min/mg dry weight. The constants for the higher concentrations are thus approximately 60-fold higher (Km) and 6-fold higher (Vmax) than those for the lower concentrations.

3. Chromatography of Accumulated Radioactive Material

Accumulation of glycerol as such, i.e., entry of glycerol into an intracellular pool without immediate phosphorylation, would be consistent with transport by either facilitated diffusion or an active transport system, and would provide evidence that transport and phosphorylation are distinct and separable reactions. To determine whether this occurs, cells were exposed to ^{14}C-glycerol and sampled at intervals. As described in Chapter II, the cells were harvested on a Millipore filter and washed immediately with cold potassium cyanide solution to inhibit further metabolism. The cells were extracted with boiling water, but the cell debris was not removed. The resulting suspension was mixed well and sampled as accurately as possible for paper chromatography (two-thirds of the total sample was used for spotting). After development of the chromatograms, the radioactive spots were counted with a Packard Model 7201 radiochromatogram scanner equipped with a Disc integrator.

Data from one experiment are shown in Table XI. Since the primary objective of this experiment was simply to determine whether glycerol was metabolized upon entry, no attempt was made to identify the products of glycerol metabolism except to compare the positions of the radioactive spots with those of glycerol itself and its initial catabolic products, glycerophosphate and dihydroxyacetone phosphate. The

amount of radioactivity counted in each spot was calculated as the percentage of the total detected. More accurate quantitative data were not considered important for the purpose of the experiment. It is apparent from the results shown in Table XI that glycerol is metabolized rapidly upon entering the cell, and that a large proportion of it is incorporated into compounds that remain at the origin. The position of each spot is given as the ratio of the distance travelled to that travelled by glycerol, i.e., as R_G. No free glycerol or glycerophosphate was detected at any time during the experiment. At 2 min, 13 per cent of the ^{14}C detected was present at a position corresponding to that of dihydroxyacetone phosphate (R_G = 0.85).

Since glycerol is a major component of bacterial lipids, it seemed likely that the material which remained at the origin might be complex molecules containing glycerol. Therefore, a second experiment was carried out in which duplicate samples were chromatographed with and without prior hydrolysis (2.0 N HCl for 1.5 hr at 100 C). There was a possibility that the cell material deposited at the origin could have occluded smaller molecules such as glycerol and prevented their movement. Therefore, chromatograms were also prepared by spotting radioactive glycerol at the origin and covering the spot with unhydrolyzed sample. These chromatograms were developed simultaneously with those containing samples alone and no retardation of movement of the glycerol was found.

The results of this experiment are presented in Table XII. In this experiment, the wash with KCN was omitted and, accordingly, a greater proportion of glycerol was incorporated into material remaining at the origin. Only at 60 min was free glycerol detected in the cells. No

TABLE XI

CHROMATOGRAPHY OF RADIOACTIVE MATERIALS ACCUMULATED BY CELLS EXPOSED TO ^{14}C-GLYCEROL

Time of sampling	R_G 0	0.05	0.09	0.14	0.20	0.23	0.31	0.85	0.95
	Percent of total radioactivity detected								
2 min	38			13	24			13	12
4 min	49				34		17		
6 min	65			35					
8 min	47	29	10	14					
10 min	69					31			
20 min	36					14			32

Standards	R_G
Glycerol	1.0
Glycerophosphate	0.46
Dihydroxyacetone phosphate	0.85

TABLE XII

HYDROLYSIS AND CHROMATOGRAPHY OF RADIOACTIVE MATERIALS ACCUMULATED BY CELLS EXPOSED TO ^{14}C-GLYCEROL

Time of sampling	R_G 0	0.12	0.19	0.23	0.30	0.34	1.0		
Unhydrolyzed	Percent of total radioactivity detected								
2 min	92		4	4					
30 min	77	7		12	4				
60 min	88						12		
	R_G 0	0.30	0.41	0.49	0.74	0.78	0.85	0.99	1.13
Hydrolyzed	Percent of total radioactivity detected								
2 min	13	21	27		28		6		5
30 min	3		6	10	6	19	17	17	22
60 min	10	1	22		23		14	18	13

Standards	R_G
Glycerol	1.0
Glycerophosphate	0.46
Dihydroxyacetone phosphate	0.85

glycerophosphate or dihydroxyacetone phosphate was found. The effect of hydrolysis indicates that the material at the origin was glycerol-containing complex molecules. The amount of such material was reduced from approximately 90 per cent of the total to approximately 10 per cent by hydrolysis, and both glycerol and dihydroxyacetone phosphate were detected in the hydrolysate.

4. Binding Protein for Glycerol

Since facilitated diffusion, according to the presently-accepted model, involves a specific binding protein located on the cell membrane, studies of several types were designed to demonstrate the presence of a binding protein in P. aeruginosa for glycerol.

a. Inhibition of Uptake by N-Ethylmaleimide

N-ethylmaleimide (NEM) has been shown to inhibit binding of sulfate ion and transport of β-galactosides in E. coli. The effect of this compound on transport of glycerol in P. aeruginosa was studied by incubating cells with NEM and measuring their ability to take up ^{14}C-glycerol after various periods of treatment.

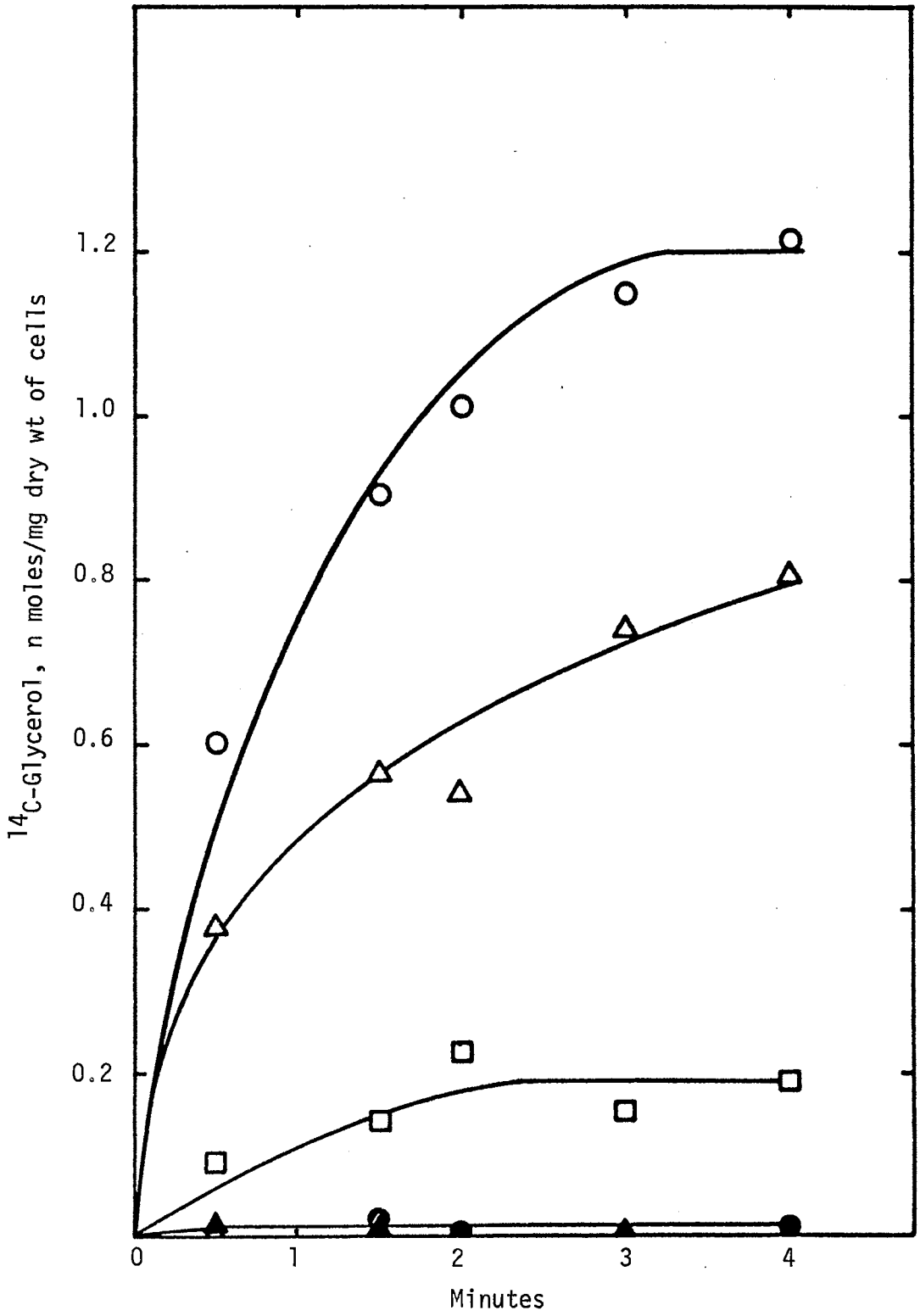
NEM was added to a suspension of induced cells in a water bath shaker at 37 C. The concentration of NEM in the flask was 1.0 mM. Mercaptoethanol was added, to samples removed at the specified intervals, to give a concentration of 5.0 mM. The time of addition of mercaptoethanol was recorded as the end of the period of reaction of NEM with the cells.

Figure 10 shows uptake curves for control cells (no NEM treatment) and for samples in which the action of NEM was stopped at 2, 8, and 15 min. A curve is also shown for cells to which was added NEM

Figure 10. Effect of N-Ethylmaleimide on Uptake of ^{14}C-Glycerol in *P. aeruginosa*.

N-ethylmaleimide was added to an induced cell suspension to a final concentration of 1.0 mM. Mercaptoethanol was added to samples removed from the reaction flask at 2, 8, and 15 min to stop the reaction with NEM. For one of the controls, NEM was mixed with mercaptoethanol immediately before being added to the cells. Measurement of ^{14}C-glycerol uptake was carried out as described in Figure 7.

Control with no NEM added (o); control with the addition of NEM and mercaptoethanol at zero time (△); 2 min NEM treatment (□); 8 min treatment (●); 15 min treatment (▲).


^{14}C-Glycerol, n moles/mg dry wt of cells
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
Minutes

mixed with mercaptoethanol immediately before mixing with the cells. There was a significant decrease in the rate of uptake in cells treated in this way, as compared with the untreated control. This may indicate that the reaction of NEM and mercaptoethanol was not complete before cells were added, or mercaptoethanol itself may have some effect on the cells. However, the inhibition of uptake in cells exposed to NEM alone was much greater. Total uptake was inhibited 83 per cent after treatment with NEM for 2 min (compared with the untreated control) and no uptake was observed with cells treated for 8 min and 15 min.

b. Effect of N-Ethylmaleimide on Glycerol Enzymes

The effect of NEM on glycerol degradative enzymes was also determined. A heavy suspension of induced cells was treated with 3.2 mM NEM for 15 minutes on a shaking water bath at 37 C, and the reaction was stopped by the addition of mercaptoethanol to a concentration of 16 mM. The cells were collected by centrifugation, and a cell-free extract was prepared. Glycerol kinase and glycerophosphate dehydrogenase were assayed and activities were 10.1 and 2.19 n moles/min/mg protein, respectively. This glycerophosphate dehydrogenase activity was approximately 38 per cent of that of untreated induced cells and glycerol kinase activity was approximately 2-fold that found with untreated cells. This may suggest that NEM affects dehydrogenase activity, but it apparently has no effect on the activity of glycerol kinase.

c. Removal of Binding Protein by Osmotic Shock

The method described in Chapter II was used to prepare "shock fluid" to be examined for the presence of a protein capable of binding ^{14}C-glycerol. The spheroplasts, which were removed by centrifugation

from the shock fluid, were used to measure uptake of ^{14}C-glycerol to determine whether the activity had been decreased by removal of binding protein. An uptake experiment using spheroplasts is shown in Figure 11. It is apparent from these data that all binding activity was not removed from the cells, but there was a significant decrease in the ability of the spheroplasts to transport glycerol. This is actually a conservative estimate of the reduction in transport activity. Not enough spheroplasts were available for an accurate determination of dry weight. The weight used in calculation was based on optical density converted to cell numbers by a curve prepared with whole cells. The number of spheroplasts was undoubtedly greater than calculated, and the uptake per cell is thus overestimated.

d. Binding Activity of Shock Fluid

The binding activity of the shock fluid was measured by equilibrium dialysis against ^{14}C-glycerol as described in Chapter II. One ml of shock fluid, containing 0.34 mg protein/ml was dialyzed overnight at room temperature against 1.0 ml of 1.6 μM ^{14}C-glycerol (total of 50681 dpm). Equilibrium was reached at approximately 16 hr. As a control, 1.0 ml of the same shock fluid which had been heated at 70 C for 5 min was placed in a second dialysis cell and treated in an identical manner. The data for this experiment, presented in Table XIII, show that the shock fluid contained binding activity for glycerol which was completely inactivated by treatment at 70 C for 5 min.

The binding activity of the shock fluid is inhibited by N-ethylmaleimide and is precipitable by ammonium sulfate. These data are shown in Table XIV. Equal portions of a single preparation of shock fluid were used in an experiment in which NEM was added to one portion

Figure 11. Uptake of ^{14}C-Glycerol by Spheroplasts Prepared by Osmotic Shock.

Induced cells were shocked by using the method for preparing "shock fluid" as described in the text. Measurement of uptake of glycerol was carried out in the same way as described in Figure 7.

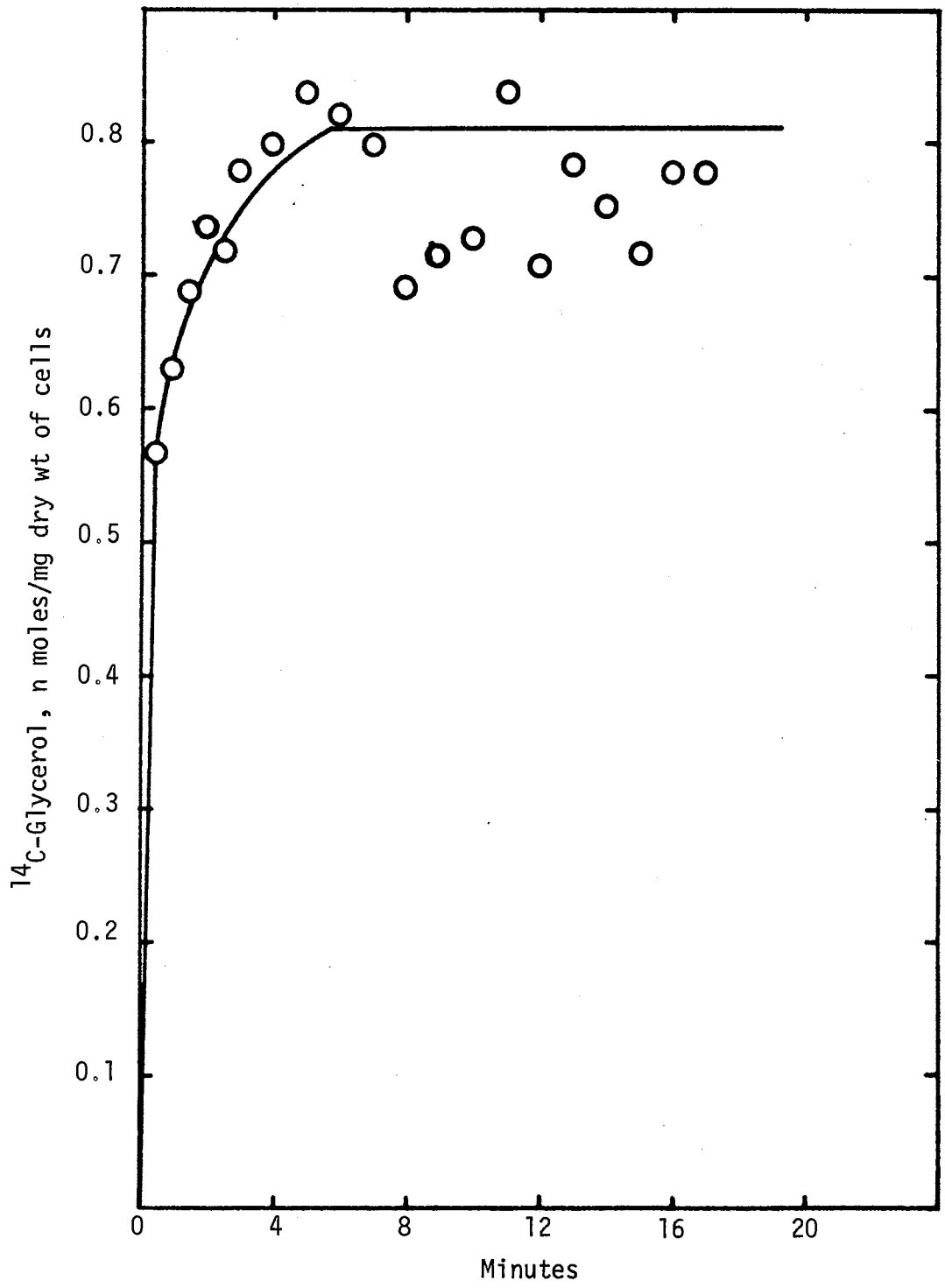

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
^{14}C-Glycerol, n moles/mg dry wt of cells
0
4
8
12
16
20
Minutes

TABLE XIII

GLYCEROL BINDING ACTIVITY IN SHOCK FLUID

Sample	Final external concentration nM	Distribution ratio cpm inside/cpm outside
Shock fluid	628.5	1.53
Heated shock fluid	799.0	0.99

The protein concentration in the shock fluid was 0.34 mg/ml.

TABLE XIV

INHIBITION OF GLYCEROL BINDING ACTIVITY OF SHOCK FLUID AND PRECIPITATED PROTEIN

Sample	Final external concentration nM	Distribution ratio cpm inside/cpm outside
Shock fluid	571.9	1.78
Shock fluid + NEM	587.1	1.02
$(NH_4)_2SO_4$ precip.	586.7	1.71
$(NH_4)_2SO_4$ precip. + NEM	799.0	0.99
Supernate from $(NH_4)_2SO_4$ precip.	811.2	0.96

Protein concentration of the shock fluid was 0.93 mg/ml.

Protein concentration of the resuspended ammonium sulfate precipitate was 0.12 mg/ml.

at a concentration of 1.0 mM. The addition of NEM completely abolished binding activity for glycerol. When ammonium sulfate was added to 85 per cent saturation, the binding activity was completely precipitated. The precipitated protein, resuspended in distilled water, was capable of binding ^{14}C-glycerol and this activity was abolished by treatment with NEM. No activity was detectible in the supernatant fluid after precipitation with ammonium sulfate.

e. Binding Activity of Transport-negative Mutant

The glycerol-negative mutant, PA-1-623, which had been shown by both methods of measuring uptake to be transport-negative was tested for glycerol-binding activity. Shock fluid was prepared and used in equilibrium dialysis, using the same techniques employed with wild type cells. The protein was also precipitated with ammonium sulfate and its binding activity determined. These data are shown in Table XV. A very small amount of binding activity was detected in both the shock fluid and the precipitated protein. This activity was destroyed by heating at 70 C for 5 min. Since the activity measured was very small, these experiments were repeated and the data are given from both experiments. Slight activity was found in both experiments, and the fact that heat treatment decreased the activity to zero indicates that the activity measured was real. Therefore, it is probable that the mutation which affects transport in PA-1-623 does not prevent synthesis of binding protein but rather causes production of a protein with almost no activity. Alternatively, the activity observed could be due to binding of glycerol to another protein, perhaps the binding protein for a compound with some structural similarity to glycerol or even an enzyme. An assay of shock fluid from the wild type cells showed that some glycerol kinase activity was present, possibly from lysis of some of the spheroplasts.

TABLE XV

GLYCEROL BINDING ACTIVITY OF TRANSPORT-NEGATIVE MUTANT, PA-1-623

Sample	Final external concentration nM	Distribution ratio cpm inside/cpm outside
Shock fluid	768.1	1.07
Shock fluid	757.1	1.10
Heated shock fluid	795.0	1.00
$(NH_4)_2SO_4$ precip.	775.6	1.05
$(NH_4)_2SO_4$ precip.	768.1	1.07

Protein concentration in the shock fluid was 0.2 mg/ml.

CHAPTER IV

DISCUSSION

From the transport studies described in Chapter III, we can conclude that the entry of glycerol into P. aeruginosa is not by simple diffusion. Induction is required for the transport of glycerol (Figures 5, 7, and 8, and Table VIII). Cells grown in medium without glycerol or glycerol phosphate (Table VIII), i.e., casamino acids medium, or glucose or lactate minimal medium, show no uptake of glycerol with the procedure of Sanno, et al. (1968) for facilitated diffusion. Cells grown on glycerol minimal medium, glycerophosphate plus casamino acids, or nutrient broth (Table VIII) or grown on glucose, then induced with glycerol (Figure 7) take up glycerol rapidly when tested either by the osmotic effect or with labeled glycerol. As mentioned in Chapter III, there may be some glycerol-containing lipids or other glycerol compounds in the nutrient broth; this could explain the induction by nutrient broth of the uptake system for glycerol.

No rapid transport (facilitated diffusion) of glycerol was observed in glucose-grown cells as measured by osmotic effect (Table VIII and Table IX). More accurate measurement using ^{14}C-glycerol showed that glycerol can diffuse slowly across the cell membrane (Figure 8). This would allow glycerol to act as inducer for the transport system or to serve as substrate for the synthesis of inducer. No induction was

observed during the period of uptake measurement. It is also possible that a very small amount of glycerol-binding protein could be synthesized endogenously in glucose-grown cells, if the transport protein is synthesized coordinately with catabolic enzymes.

The uptake of glycerol by cells grown on glycerol plus glucose indicated no apparent effect of glucose (Figure 6 and Table VIII) on synthesis of the transport protein. This indicates that glycerol transport is not subject to catabolite repression by glucose in P. aeruginosa. The opposite effect, complete repression of synthesis of the transport system, was found with E. coli and Achromobacter sp. (Figure 6 and Table VIII).

The stable kinaseless mutant, which is required to determine whether glycerol or glycerophosphate or both can act as the true inducer for the entire glycerol catabolic system, has not been isolated. Results obtained in the present study show that either glycerol or glycerophosphate can act as the inducer for glycerol transport in the wild type, though the cells cannot utilize glycerophosphate as sole carbon source.

In experiments using labeled glycerol, cells induced with glycerol showed rapid transport of glycerol which reached a maximal accumulation at 2.5 min (Figure 7). Kinetic studies of glycerol uptake yielded non-linear double reciprocal plots (Figure 9), i.e., two distinct Km values for transport of high and low concentrations of glycerol. The occurrence of two distinct apparent affinity constants (Km) in transport systems has been reported by Ames (1964) for histidine transport by S. typhimurium, Halpern and Even-Shoshan (1967) for glutamate transport by E. coli, Rotman and Radojkovic (1964) for galactose uptake in

E. coli, Pall (1969) for amino acid transport by N. crassa, and Kay and Gronlund (1969) for proline transport by P. aeruginosa. Halpern and Even-Shoshan (1967) proposed that the glutamate permease of E. coli is an allosteric protein, and that the binding of glutamate to the allosteric site can reduce the activity of the permease. Ames (1964) reported that histidine can enter into S. typhimurium either by a transport system specific only for histidine or by a permease for aromatic amino acids. Rotman and Radojkovic (1964) indicated that two distinct Km values for galactose transport in E. coli are due to transport of galactose by two different transport systems. Furlong and Weiner (1970) have shown that leucine is transported in E. coli by two distinct systems with Km values of 0.2 and 2 μM, respectively. One is probably specific for leucine while the other probably transports leucine, isoleucine, or valine.

Whether an allosteric effect or a second low affinity permease can explain the kinetics observed in the present study is not known at present. The ratio of values of Vmax for high and low concentrations of glycerol (approximately 6) is almost the same as that reported by Kay and Gronlund (1969) for proline transport in P. aeruginosa (approximately 5). These authors were also not able to offer a definite explanation for the kinetics observed. In view of the large difference in Km values (60-fold) for the two concentration ranges, it seems quite possible that glycerol may be able to enter the cell by two transport systems, one specific for glycerol and the other possibly a polyalcohol or other transport system with a low affinity for glycerol.

The rapid entry of a substrate into bacteria may be due to either facilitated diffusion or an active transport system. All the available

data indicate that active transport across a biological membrane involves, first, a substrate-specific carrier which facilitates movement across the permeability barrier, and second, an energy-coupled mechanism which produces a net accumulation of substrate in the cell against a concentration gradient. In facilitated diffusion, membrane carriers catalyze the equilibration of intracellular and extracellular substrate concentration, without the consumption of energy by the cell.

Two kinds of experiments can be utilized to distinguish between the two systems. After the prediction of the phenomenon by Widdas in 1952, Park, et al. (1956) and Rosenberg and Wilbrandt (1957) devised the counterflow experiment to demonstrate facilitated diffusion. Park, et al. (1956) showed that the addition of a high concentration of glucose to red blood cells, which had been equilibrated with xylose in a balanced salt solution containing xylose, caused the transient net movement of xylose out of the cell against a concentration gradient. This type of experiment also can be applied to an active transport system in bacterial cells, using energy uncouplers to allow measurement of the activity of the membrane carrier only (Cohen and Monod, 1957; Horecker, et al., 1960; Koch, 1964). When the cells are "poisoned" with energy uncoupler, the function of the membrane carrier remains intact although movement against a gradient is no longer possible.

Another type of experiment involves determining the effect of energy uncoupling upon entry into the cell (rather than loss of accumulated substrate). The present studies included only the latter type of experiment. Studies using both labeled and unlabeled glycerol (Table IX and Figure 7) favor facilitated diffusion as the mechanism for glycerol transport in P. aeruginosa. The presence of the energy

inhibitors, sodium azide, 2,4-dinitrophenol, and potassium cyanide, had no effect on glycerol uptake, even when cells were preincubated for more than 30 minutes with the inhibitors. These experiments indicate that enery is not required for glycerol transport in P. aeruginosa.

A membrane-associated transport protein or binding protein is thought to be involved in both facilitated diffusion and active transport. Lysozyme treatment or osmotic shock can release some membrane-bound enzymes and transport factors (Heppel, 1967). The osmotic shock procedure was selected for the present study. The spheroplasts formed from osmotically shocked cells still exhibited ability to take up glycerol (Figure 11); however, there was a significant decrease in glycerol transport. In other words, partial removal of the membrane carriers was achieved. Reductions of 25 to 70 per cent have been reported with osmotically shocked cells for transport of arginine, leucine and lysine (Wilson and Holden, 1969) and for galactose (Anraku, 1967). The removal of a glycerol-binding protein by osmotic shock was also demonstrated by equilibrium dialysis studies with the shock fluid and with protein precipitated from the shock fluid with ammonium sulfate (Tables XIII and XIV). Heat-inactivated shock fluid served as the control. These data also suggest that there is a transport protein for glycerol in the shock fluid.

N-ethylmaleimide has been reported to react rapidly and specifically with sulfhydryl groups (Freidmann, et al., 1949, 1952). However, it has also been shown to react with the amino group of peptides, with imidazole and with cysteine (Smyth, et al., 1960). Fox and Kennedy (1965) first used NEM to reduce the number of carrier sites for a transport system. Studies of transport with NEM-treated cells (Figure 10)

show that NEM does affect the transport of glycerol in P. aeruginosa. A two-minute incubation of cells with NEM decreased the transport of glycerol to 17 per cent of that in the untreated control. Eight-min and 15-min treatments of cells with NEM completely eliminated glycerol uptake. The effect of NEM could be explained in two ways. First, NEM could react irreversibly with membrane carrier to prevent the transport of glycerol. Second, NEM could inactivate the glycerol catabolic enzymes, causing an accumulation of metabolic intermediates which might possibly prevent uptake. L-α-glycerophosphate dehydrogenase from rabbit muscle contains 9 to 11 SH groups per protein molecule (Telegdi and Keleti, 1964). Two histidine residues at the active center of the L-α-glycerophosphate dehydrogenase have been reported to be critical for activity of this enzyme (Apitz- Castro and Suarez, 1970). Sensitivity of L-α-glycerophosphate dehydrogenase to sulfhydryl reagents, e.g., p-chloromercuribenzoate (Van Eys, et al., 1959; Telegdi and Keleti, 1964), thiazole adenine dinucleotide (Van Eys, et al., 1962). 1-hydroxy-3-iodo-2-propanophosphate (Hartman, 1968) and N-alkylmaleimides (Anderson, et al., 1970) suggests that sulfhydryl groups of the enzyme may react with sulfhydryl reagents to cause a conformational change of the enzyme, resulting in inactivation. In the present study, extracts were prepared from cells treated with NEM and enzyme assays were performed. The L-α-glycerophosphate dehydrogenase activity of the extract from cells treated for 15 min with NEM was decreased to about 38 per cent of that in an extract from untreated induced cells. This may suggest that the reaction of NEM with dehydrogenase could cause inhibition of the enzyme activity and accumulation of L-α-glycerophosphate, and that this might decrease the total accumulation of radioactivity.

However, there is no reason to believe that transport would be completely eliminated for this reason, and the cells treated for the same, or a shorter, period of time with NEM exhibited no transport of glycerol (Figure 10). Also, equilibrium dialysis studies of NEM-treated shock fluid showed that NEM prevented binding of labeled glycerol when the shock fluid was dialyzed against ^{14}C-glycerol (Table XIV). We can conclude that NEM does have some effect on the dehydrogenase and this may indicate that sulfhydryl groups are essential to the activity of the P. aeruginosa enzyme. However, the major inhibitory effect of NEM on transport itself is due to the reaction of NEM with the binding protein.

Chromatographic separation of compounds accumulated in wild type cells exposed for various times to labeled glycerol, showed that strain PA-1 incorporated glycerol primarily into glycerol-containing compounds and did not accumulate free glycerol (Tables XI and XII). Hydrolysis of the material produced a number of more rapidly moving compounds, one of which was located in the same position as the glycerol standard. Glycerol is known to be incorporated into bacterial lipids as glycerol or glycerophosphate. Mindich (1970) reported that glycerol is incorporated primarily into lipid in B. subtilis and that very little free glycerol is found in the cells. Hayashi and Lin (1965a) found that all of the radioactive material recovered from an extract of dehydrogenase-less mutant cells, incubated 5 min with ^{14}C-glycerol, was chromatographically indistinguishable from L-α-glycerophosphate; no free glycerol was recovered.

Glycerol transport was studied in a number of mutants by the method based on osmotic effect (Table X). Only two, PA-1-93 and PA-1-623, showed no facilitated diffusion of glycerol. One of these mutants,

PA-1-623, was further studied by measuring ^{14}C-glycerol uptake and the binding activity of shock fluid. Only slight accumulation of glycerol was observed in this mutant (Figure 8). Almost no binding activity was found in the shock fluid (Table XV).

Until recently, the belief has been widely held and frequently stated that apparent transport and accumulation against a gradient can be achieved by phosphorylation of a molecule which enters the cell by simple diffusion. This was based on the idea that the membrane is much more impermeable to phosphorylated (charged) compounds than to small neutral molecules. It was this mechanism which was first proposed for the capture of glycerol in E. coli by Hayashi and Lin (1965). According to this model, glycerol kinase activity would be essential for accumulation of glycerol within the cell and the kinase would be equivalent in effect to a "permease." More recent information (Kaback, 1970) supports the idea that "the passive diffusion properties of the membrane with regard to free sugar and sugar-phosphate appear to be rather similar." Therefore, "phosphorylation does not appear to be a mechanism for 'trapping' sugar." It seems reasonable to assume that this statement is applicable in the case of glycerol and, indeed, Sanno, et al. (1968) reported inducible facilitated diffusion of glycerol in E. coli.

Nevertheless, it would be desirable to obtain data which would clearly distinguish between trapping of diffused molecules by phosphorylation and a specific mechanism for transport. The fact that the transport system is inducible does not offer sufficient proof of the transport mechanism since the kinase is also inducible. The best possible evidence for the independence of transport and phosphorylation would be demonstration of each activity in a mutant lacking the other activity,

i.e., the demonstration of an inducible transport system in a kinaseless mutant and of inducible kinase activity in a transport-negative mutant. Kinase activity in a transport-negative mutant, PA-1-623, was demonstrated and the lack of accumulation of glycerol was correlated with the absence of glycerol-binding protein. Only one mutant isolated in these studies (PA-1-812) appeared to be defective in kinase activity, according to enzyme assays with cell extracts (Table V). Accordingly, this mutant was grown on glucose, induced with glycerol and used for measurement of uptake of ^{14}C-glycerol. A portion of the same cell suspension was used to prepare cell-free extract for enzyme assays to determine whether kinase was absent in these cells. This was necessary because of high rate of reversion to wild type in this mutant. The results of these enzyme assays were shown in Table V and the uptake data were not presented because it was apparent from the enzyme levels found that most of the cells in the population were revertants. Uptake of labeled glycerol was similar to that in the wild type culture. The instability of this mutant made it impossible to demonstrate transport in the absence of kinase activity, but the data for mutant PA-1-623 show that simple diffusion followed by phosphorylation cannot account for the rapid uptake of glycerol observed with the wild type cells. These data are more conclusive than those presented by Sanno, et al. (1968) in support of a facilitated diffusion system for glycerol transport in *E. coli*.

The control of synthesis of the catabolic enzymes for glycerol in *P. aeruginosa* is similar to that in *E. coli* in some respects and different in others. Glycerol kinase and L-α-glycerophosphate dehydrogenase are inducible enzymes in *P. aeruginosa* (Table I) as in *E. coli*. This was also observed by Cowen (1968). The low enzymatic activity of kinase

and dehydrogenase in nutrient broth-grown cells may indicate the presence of glycerol-containing compounds in nutrient broth; however, the amount apparently is not high enough for full induction of enzyme activity. Synthesis of glycerol kinase and L-α-glycerophosphate dehydrogenase is not subject to catabolite repression in P. aeruginosa (Table I), whereas in E. coli synthesis of both of these enzymes and the active transport system for L-α-glycerophosphate is very strongly repressed by glucose (Koch, et al., 1964). The active transport system for L-α-glycerophosphate, which is a part of the glycerol regulon in E. coli (Cozzarelli, et al., 1968), is absent in P. aeruginosa.

The question of whether glycerol or L-α-glycerophosphate acts independently as inducer in P. aeruginosa can be answered only by the use of mutants, either one completely lacking glycerol kinase activity or one producing a cross-reacting protein with very low glycerol kinase activity, as used in E. coli by Lin and his coworkers (Koch, et al., 1964; Hayashi and Lin, 1965b). The kinaseless mutant, PA-1-812, which was isolated in the present study, failed to remain stable (Table V) and thus it was impossible to determine the true inducer for P. aeruginosa. Data obtained with this mutant and with PA-1-623 indicate, however, that endogenously formed L-α-glycerophosphate may be the true inducer for both enzymes, even though exogenous L-α-glycerophosphate apparently cannot enter the cell to act as inducer. Both enzyme activities in an extract of cells grown on nutrient broth plus glycerol phosphate were only slightly increased as compared to those of nutrient broth-grown cells. This indicated that exogenous L-α-glycerophosphate cannot cross the cell membrane and initiate the induction of the kinase and dehydrogenase. The failure of P. aeruginosa to grow on glycerophosphate also

indicates that the cell is not permeable to this compound. The fact that reversion of PA-1-812 can lead to recovery of both enzyme activities may indicate that L-α-glycerophosphate is the actual inducer (Table V).

Since PA-1-812 reverts, it can be assumed to be a single site mutant. There are several possible ways in which a single site mutation could affect both enzyme activities. The mutation could be of the reading-frame type or it could be an extreme polarity mutation. If a frame-shift were to be invoked as explanation of the effect on two genes, it would be necessary to assume that they are contiguous, or that they are closely-linked and not separated by genes coding for another necessary function. This could also explain the relation between glycerol catabolic mutants and phage sensitivity if it were assumed that the locus for phage resistance is contiguous to the loci for the kinase and the dehydrogenase. Two facts argue against this explanation. Mutants of the PA-1-92 series and the PA-1-623 series are apparently deletion mutants since they do not revert. None of these mutants (of those tested) lack kinase activity and, since the deletion is apparently quite extensive, if the kinase locus were contiguous to the loci for phage adsorption and for the dehydrogenase, it might be expected to have been affected by a deletion. Secondly, all mutants of the PA-1-800 series, all of which revert, are resistant to phage Pø-6. It is highly unlikely that they are all of the frame-shift type. The same arguments can be used with reference to an extreme polarity mutation. Both of these types of mutation would be most likely to affect the two glycerol enzymes without affecting phage sensitivity, unless the locus for phage adsorption is a part of the glycerol operon. This might be possible if

glycerol is involved in the phage adsorption site.

A second possible explanation for the effect of a single site mutation on two enzymes might be that the mutation involves the control system, e.g., a mutation in the promoter or in the regulator gene resulting in production of a "super repressor" (non-inducibility mutation). A promoter mutation would involve the same assumptions as to linkage of the loci as were necessary for explanations involving frame-shift and polarity mutations. However, contiguity of the loci would not be necessary in the case of a mutation of the R^S type, if all of the loci for the glycerol catabolic enzymes are controlled by a single repressor. This possibility cannot be eliminated without genetic data on the locations of the loci in question and the site of the mutation in PA-1-812.

The third explanation is the most probable one. If L-α-glycerophosphate is the inducer for both the dehydrogenase and the kinase, no dehydrogenase would be synthesized in the absence of kinase activity when glycerol is used as inducer. Therefore, if the mutation in PA-1-812 is located in the structural gene for the kinase, rendering the enzyme inactive, synthesis of both dehydrogenase and kinase would be dependent upon reversion to the wild type which would allow formation of the inducer from glycerol. This explanation is also supported by the enzyme data for one of the deletion mutants, PA-1-623, which has no dehydrogenase activity. In this and similar mutants (Table V), kinase activity is approximately twice as high as in the wild type. This could be due to accumulation of inducer in the absence of the dehydrogenase, if L-α-glycerophosphate is the true inducer. Cozzarelli, et al. (1968) attributed elevated kinase activity in a dehydrogenaseless mutant

to "more complete neutralization of the repressor by the higher intracellular pool of L-α-glycerophosphate."

Glycerol is a major component of the cell wall of P. aeruginosa (Adams, et al., 1967; Bobo and Eagon, 1968), and the synthesis of glycerol, which usually involves the same intermediates as its catabolism, should be necessary for growth on any other carbon source such as glucose or lactate. Therefore, the complete absence of glycerol dehydrogenase activity in the wild type grown on lactate, and the loss of this enzyme in mutants such as PA-1-623, suggest that there may be two glycerophosphate dehydrogenases in P. aeruginosa. The one which was measured in the present study is NAD-independent and catalyzes the catabolic conversion of glycerophosphate to dihydroxyacetone phosphate. A second dehydrogenase could be NAD, NADP, or FAD-dependent and could catalyze the reverse reaction for the synthesis of lipids. Kito and Pizer (1968, 1969) discovered and purified a glycerophosphate dehydrogenase from a glucose-grown E. coli mutant which lacks the catabolic dehydrogenase. This dehydrogenase required a puridine nucleotide, NADPH or NADH, as cofactor for enzyme activity. Kistler, et al. (1969) also found an FAD-dependent glycerophosphate dehydrogenase in E. coli which apparently functions only for anaerobic catabolism of glycerol. In the present study, enzyme assays of cell-free extracts of PA-1 grown on nutrient broth or induced with glycerol, using Kito and Pizer's method (1968), found no evidence of NAD or NADP-linked dehydrogenase activity. Therefore, if P. aeruginosa possesses a specific anabolic glycerophosphate dehydrogenase, it is not similar to that found in E. coli. If there is no pathway for synthesis of glycerol distinct from the catabolic pathway, a revertant would have a strong selective

advantage in growth on any type of medium. This would be consistent with the high percentage of revertants frequently found in cultures of glycerol mutants in the present study.

A high triose phosphate isomerase activity in cells grown on either glucose or glycerol minimal medium might suggest that there are two isomerases, one, induced by glycerol, catalyzing the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate, and the other induced by glucose specifically for the reversal of that reaction (Cowen, 1968). However, high enzyme activity observed during growth on lactate (Table I), which is not degraded by the glycolytic route, would exclude the above hypothesis and suggest constitutive synthesis of this enzyme. The isomerase activity in nutrient broth-grown cells is lower compared to that of cell-free extract from cells grown in minimal medium (Table I). An inhibitory effect of an accumulated compound on isomerase activity could account for this finding, or isomerase synthesis could be subject to catabolite repression. Alternatively, since the concentration of growth factors in nutrient broth is sufficient to provide rapid growth, this may cause a decrease in synthesis of this enzyme, which is not so necessary in nutrient broth. None of these possibilities can be excluded since no data are available for metabolic control of this enzyme. Low isomerase activity in the group of non-reverting mutants may explain the slow growth of these mutants on glucose-methionine medium (Table IV and Mose, 1970). The low isomerase activity of these mutants may suggest that the deletion postulated for these mutants may involve partial deletion of the structural gene for the isomerase. The over-accumulation of L-α-glycerophosphate, which is due to the elevated glycerol kinase activity in these mutants may also

possibly inhibit isomerase activity by competing with the substrate (dihydroxyacetone phosphate or glyceraldehyde-3-phosphate) and thus could retard growth. Cozzarelli, et al. (1965) found that mutants lacking dehydrogenase are subject to growth inhibition in the presence of glycerol or L-α-glycerophosphate. They suggested that this might be due to competition of L-α-glycerophosphate with other phosphorylated substrates, thus inhibiting other enzymatic reactions. Further study of the inhibitory effect of L-α-glycerophosphate on the isomerase in glucose-grown cells of the mutants will be required to clarify this problem.

The inhibitory effect of glucose on the utilization of glycerol by P. aeruginosa was observed by Cowen (1968). He proposed that the enzymes for the glycerol degradative pathway are synthesized normally but are reversibly inactivated in the presence of glucose or its intermediates. This suggestion was based on the rates of utilization of glycerol after glucose was exhausted from the medium. Data obtained in the present study with cell-free extracts of cells grown on glucose plus glycerol or induced in the presence of glucose (Table I) agree with his hypothesis. The 2.5-hr induction time allowed for induction of glucose-grown cells in the glucose-glycerol mixture, compared to the 4-hr induction time used for glycerol alone, may explain the lower enzyme activities in the former (Table I). Zwaig and Lin (1966) reported that fructose-1, 6-diphosphate is an inhibitor of glycerol kinase and causes catabolite inhibition in the presence of glucose. Further studies, testing the intermediates of the Entner-Douderoff pathway (see Figure 1), which is the predominant route of glucose catabolism in P. aeruginosa, may reveal the basis for the inhibitory effect of glucose in P. aeruginosa.

A group of mutants, the PA-1-800 series, was isolated in attempts to obtain a kinaseless mutant for study of the actual inducer and to obtain phage-sensitive mutants for use in determining linkage between the loci for inability to use glycerol, resistance to phage and methionine auxotrophy. However, these mutants showed a great deal of variation in growth on glycerol (Tables II and III). From repeated growth studies, it is certain that they are defective in glycerol metabolism although their reversion rates are quite high. Some of the variation in growth of these mutants could be due to the accumulation of toxic levels of α-glycerophosphate. This would also exert a strong selective pressure in favor of wild type revertants in the population. The sensitivity of this group of mutants to phage Pø-6 was recovered when reversion occurred; therefore, the turbid plaques formed on these cells (Table VI) could be caused by the presence of a large proportion of wild type revertants in the population. These data seem to indicate that the relation between phage resistance and inability to grow on glycerol is based on involvement of glycerol in the phage adsorption site rather than on a defect in two separate functions occurring as the result of a single mutation. Other possibilities were discussed above.

The sensitivity to phage Pø-5 was sufficiently stable to allow preparation of plate stocks of these mutants for use in transduction. Again, the reversion rate of the methionine auxotrophs was very high and the ability to make methionine in the colonies tested (Table VII) could have been the result of reversion rather than transduction of the $meth^+$ locus. However, the inability to use glycerol must have resulted from transduction of gly^- from glycerol-negative mutants. In only one case was co-transduction of the two markers possibly achieved (Table VII).

If co-transduction did occur, it indicates the close linkage of these two genes. Isolation of non-reverting mutants for use in transduction is still required before linkage can be definitely established. Phage Pø-5 may be useful in such studies, if resistance to phage F116 (the phage commonly used for transduction in P. aeruginosa) commonly accompanies mutations in the glycerol pathway. Phage Pø-5 had not been shown to be capable of transduction prior to its use in the present investigation.

CHAPTER V

SUMMARY AND CONCLUSIONS

The transport of glycerol in P. aeruginosa, strain PA-1, was found to require induction by glycerol or glycerophosphate. No energy is involved in this transport system; that is, glycerol enters the cell by facilitated diffusion. The synthesis of the glycerol transport system is not subject to catabolite repression in the presence of glucose.

Measurements of uptake using ^{14}C-glycerol showed that intracellular glycerol reaches a maximum at 2.5 min. Kinetic studies suggested that there may be two systems for glycerol transport, one with high affinity for glycerol, the other with low affinity.

Binding activity for glycerol was decreased by osmotic shock. This activity could be detected, by equilibrium dialysis, in the "shock fluid," i.e., the supernatant fluid from osmotically shocked cells. The protein nature of the glycerol-binding material was demonstrated by its inactivation in 5 min at 70 C, precipitation of the active material with ammonium sulfate, and complete inhibition of its activity by the sulfhydryl reagent, N-ethylmaleimide. N-ethylmaleimide also inhibited transport of glycerol by whole cells. The binding protein was absent in shock fluid prepared from a transport-negative mutant.

Chromatography of radioactive compounds accumulated by cells exposed to ^{14}C-glycerol indicated that P. aeruginosa incorporated

glycerol primarily into complex molecules, probably glycerol-containing lipids. Glycerol, dihydroxyacetone phosphate and other unidentified radioactive products were formed on hydrolysis in 2 N HCl.

Enzyme assays, using cell-free extracts of the wild type and several glycerol-negative mutants which had been grown or induced with various carbon sources, showed that glycerol kinase and L-α-glycerophosphate dehydrogenase are inducible in P. aeruginosa. Indirect evidence indicates that endogenously formed L-α-glycerophosphate may be the true inducer of these enzymes. Exogenous glycerophosphate cannot serve as inducer. Triose phosphate isomerase is constitutively synthesized. Glycerol degradative enzymes, like the transport protein, are not subject to catabolite repression in the presence of glucose.

Two groups of glycerol-negative mutants were used for the present study. One group of deletion mutants was found to have no glycerophosphate dehydrogenase activity, elevated glycerol kinase activity and low isomerase activity. These mutants were also transport-negative. The presence of glycerol kinase in the absence of transport shows that glycerol is not simply "trapped" in the cell by phosphorylation. Rather, transport and phosphorylation are distinct activities, coded for by genetically separable loci.

A second group of mutants, isolated in the present study, had high reversion rates and their enzymatic lesions could not be readily identified. One mutant of this group lacked glycerol kinase and had very low glycerophosphate dehydrogenase activity. Reversion restored both activities. Several possible explanations for this effect were suggested, but the most probable is that endogenously formed L-α-glycerophosphate is required for induction of both enzymes. The elevated kinase activity

in mutants completely lacking dehydrogenase activity supports this conclusion.

Mutants of this group, like the deletion mutants, are phage-resistant, but sensitivity to phage is regained when reversion to wild type utilization of glycerol occurs. This indicates that glycerol may be involved in the adsorption site for some Pseudomonas phages. Using a different phage, to which these mutants are sensitive, it was possible to demonstrate transduction of the gly^- locus.

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