# CHARACTERIZATION OF BINDING AND TRANSPORT OF GLYCEROL BY PSEUDOMONAS AERUGINOSA

By

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# CHARACTERIZATION OF BINDING AND TRANSPORT OF GLYCEROL BY PSEUDOMONAS AERUGINOSA

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To my husband, Harold, I am dedicating this thesis, for it was through his understanding, encouragement, and cooperation that I was able to realize this goal.

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## INTRODUCTION

Bacterial transport involves the selective permeation of the cell membrane by certain molecular species, allowing them to enter the cell. These transport systems have, to date, proven to be functionally specialized and distinct from metabolic enzymes, although they seem to operate in conjunction with such enzymes. Thus, it is thought that the entry of most organic nutrilites metabolized by a bacterium must be mediated by some type of permeation system.

The fundamental importance of the transport phenomenon has long been recognized in animal physiology. However, microbiologists viewed data supporting bacterial systems with doubt. As early as 1939, Deere, et al. (1939) described a strain of Escherichia coli which did not ferment lactose but did display all the necessary enzyme activities. Since the metabolism of all other carbohydrates by this strain was unaffected, the logical assumption to be made was that each cell contained a multitude of specific permeation systems, each for a separate substrate. At that time, no positive supportive evidence existed and no direct experimental approach seemed open. A more convincing example of crypticity was reported by Douderoff and his co-workers (1949, 1951, 1956). A mutant strain of E. coli was unable to metabolize glucose although it could completely degrade maltose. Since maltose is split to form glucose, and in this way is metabolized, a conclusion providing

for the presence of a stereospecific permeation system had to be considered.

From 1955 to 1960, several investigators (Cohen and Rickenberg, 1955a; Rickenberg, et al., 1956; Kepes and Monod, 1957; Jacob, et al., 1960), working with E. coli, extended the earlier observations on lactose by demonstrating the requirement of an inducible protein for transport of this compound and certain other β-galactosides. This protein was proven to be distinct from both β-galactosidase and transacetylase but was controlled jointly with them under a single regulatory mechanism, the operator region of the lactose operon. Since the function and genetics of the lac operon have been discussed in detail elsewhere (Jacob and Monod, 1961; Vogel and Vogel, 1967; Beckwith and Zipser, 1970) they will not be further elucidated here. However, inasmuch as investigations on the galactoside permeation system have yielded a wealth of information which has led to the formulation of many transport models, this subject will be discussed in more detail.

Because the action of the galactoside transport protein was considered to be catalytic, it and all other similar proteins received the name permease (Rickenberg, et al., 1956), a term which has since been discussed and criticized. In 1965, Fox and Kennedy isolated a membrane-associated protein with properties similar to those of the β-galactoside permease. However, since this protein has not been indisputably proven to be identical with the "galactoside permease", it has been called the M (membrane) protein.

Within the period 1955 to 1960, the β-galactoside permeation system was found to transport such other compounds as melibiose and phenyl-β-D-thiogalactoside as well as lactose and thiomethylgalactoside

(TMG). The latter compound proved to be particularly useful since it caused induction but could not be metabolized (Rickenberg, et al., 1956). Investigations utilizing TMG proved galactoside uptake to be an active process whereby TMG could be recovered unchanged within the cell (Sistrom, 1958), and accumulation of the compound against a gradient could be inhibited by such uncoupling agents as 2,4-dinitrophenol or sodium azide. Also, the latter phase (or plateau value) of a plot of compound accumulated vs. time, the active transport curve, was interpreted to reflect the balance between continued uptake and equivalent efflux of the radioactive substrate. At this time in the uptake sequence, the ratio of the concentration of TMG inside the cell to that in the medium was calculated to be between 50:1 and 500:1. Furthermore, it was noted that addition of the previously-mentioned uncouplers during this plateau period caused an immediate decrease in the intracellular concentration of TMG.

By 1960, other permeation systems in addition to that for $\beta$-galactosides had been proposed: exploratory research had been done on amino acid uptake (Gale, 1947, 1954; Cohen and Rickenberg, 1955b, 1956), on accumulation of citrate and other Krebs cycle intermediates (Kogut and Podoski, 1953; Barrett, et al., 1953; Gilvarg and Davis, 1954), on tartaric acid permeation (Shilo and Stanier, 1957) on glucuronide transport (Stoeber, 1957), and on several other carbohydrate systems. However, the $\beta$-galactoside research was by far the most sophisticated and, by 1960, it provided the necessary kinetic data for postulation of the first detailed transport model (Kepes, 1960).

This model assumed the presence of two components, a mobile carrier and the permease. The mobile carrier was described as a

constitutive, nonspecific protein which could exist in an energized state (*carrier) or in a low-energy complexed state (carrier-substrate). The permease was specific, with its function being catalysis of reaction (1).

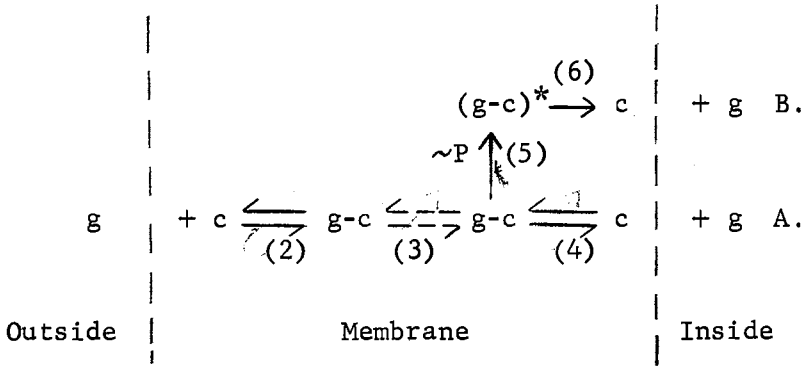
$$*\text{Carrier} + \text{Substrate} \xrightarrow[\text{Energy Dissipation}]{\text{Permease}} \text{Carrier-Substrate Complex} \quad (1)$$

As illustrated, the energy involvement in this system was with the formation of the initial complex at the outer surface of the membrane. A later interpretation was presented by Koch (1964). The major difference was that in Koch's model, energy was involved with the dissociation of substrate from carrier at the inner face of the membrane. A study reported by Winkler and Wilson (1966) supported Koch's hypothesis on energy involvement, but their experiments with sodium azide, iodoacetate, and 2,4-dinitrophenol indicated that the carrier was the permease itself, and that it was involved in active transport as well as in the facilitated diffusion seen in energy-poisoned cells.

A number of models for transport have since been proposed; the three most recent ones are shown below in reactions 2-15 and 18-30. Each depicts three general steps as necessary components of the transport mechanism: (1) association of a carrier (permease) with substrate; (2) translocation by some unknown method, possibly rotation, vibration, or configurational change; and (3) dissociation of substrate inside the cell.

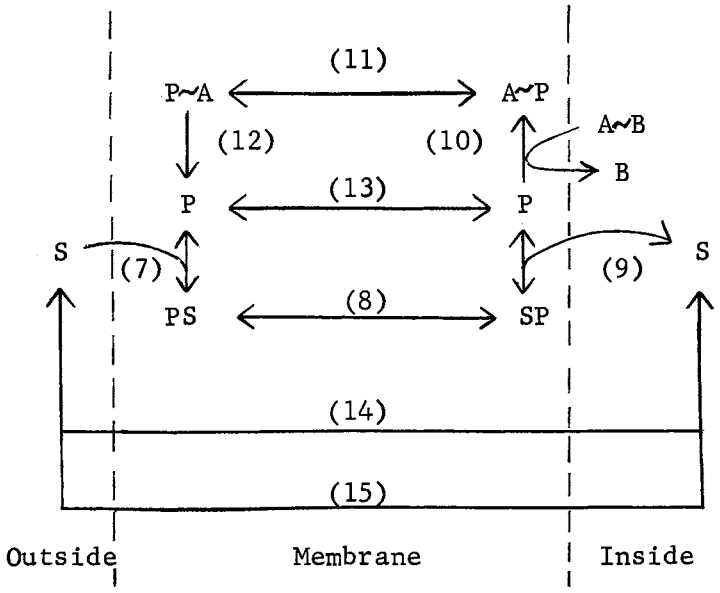
The first and least elaborate model (reactions 2-6) was presented by Manno and Schacter in 1970. It simply noted two pathways, A and B,

by which $\beta$-galactosides could be transported. Influx via A, illustrated by reactions 2,3, and 4, represents transfer by facilitated diffusion. When pathway B is used (reactions 2,3,5,6), energy coupling is involved, converting the carrier-galactoside complex (g-c) to an altered form (g-c*), which then is dissociated in reaction 6, yielding free carrier and free internal galactoside. Energy coupling retains intracellular galactoside by recycling it through reactions 4,5, and 6. The authors state that they have omitted the representation of unloaded carrier translocation for the sake of simplicity.




(2-6)

For clarity, this author has added the boundaries of the cell membrane and slightly altered the orientation of the model represented by reactions 7 through 15 (Kepes, 1971).




(7-15)

This model is much more complicated than the previous one and requires several specific assumptions. First, energy coupling is irreversibly involved with formation of an energy donor-permease complex (reaction 10), this being represented by A~P. Second, at least two steps are associated with energy coupling, the second being the breakage of the covalent bond of P~A (energy dissipation, reaction 12). Third, permease can exist in a free form (P), as a substrate complex (PS), or in an energized form (P~A), all of which can be oriented with the active site inward or outward. This model hypothesizes not only an efflux channel inhibitable by uncoupling agents (15) but a nonspecific leak channel (14).

Facilitated diffusion, in this model, involves reactions 7, 8, 9, and 13, whereas inward active transport follows the pathway 7, 8, 9, 10, 11, 12. Outward-oriented P forms a complex with substrate (S). The complex is translocated to inward-oriented PS which can only accumulate S on the inner face by formation of a second complex (inward-oriented A~P) requiring energy. Conversion to outward-oriented P~A is followed by the energy dissipation step (reaction 12) which reforms outward-oriented P. Kepes states that the fate of the radical, A, is disregarded due to lack of experimental evidence. Efflux can occur via several pathways. When substrate is removed from the medium causing the cell to lose substrate in order to maintain equilibrium, channels 14 and/or 15, being independent of the saturable permease protein, might be utilized. A permease-mediated exit could be accomplished if outward-oriented P were converted to inward-oriented P by the reverse of reaction 13, this combining with S at the inner face (reverse of reaction 9) and then following the reverse reaction

sequence through reactions 8 and 7. At the steady state in facilitated diffusion, reactions 7, 8, and 9 occur at equal rates in the inward and outward directions, simply bringing about exchange. During the steady state in active transport, substrate still enters through reactions 7, 8, 9, 10, 11, 12 but leaves partly through reversal of reactions 9, 8, and 7 and partly through 14 and 15, the flux through 10, 11, and 12 counter-balancing that directed through 14 and 15.

With the discovery of the phosphoenolpyruvate-phosphotransferase system in E. coli, by Kundig, et al. (1964), a new mode of substrate entry was established. In this instance, several proteins are involved (as indicated in reactions 16 and 17) and the sugar substrate is trapped as a phosphorylated derivative upon entry.

$$\text{PEP} + \text{HPr} \xrightarrow{\text{Enz I, } Mg^{++}} \text{P-HPr} + \text{Pyruvate} \qquad (16)$$

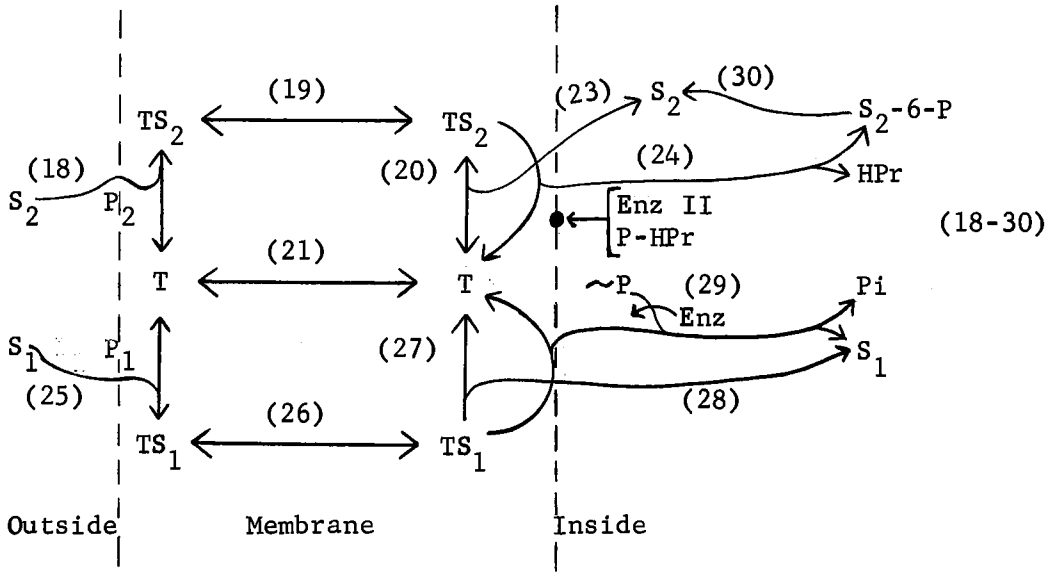
$$\text{P-HPr} + \text{Sugar} \xrightarrow{\text{Enz II, } Mg^{++}} \text{Sugar-P} + \text{HPr} \qquad (17)$$

In the first reaction (16), the phosphoryl group is transferred from phosphoenolpyruvate (PEP) to the histidine moiety of a heat-stable, low molecular weight protein (HPr) upon catalysis by enzyme I. This first enzyme is soluble, constitutive, and nonspecific with respect to sugars (Kundig, et al., 1966, Simoni, et al., 1968). In the second reaction (17), the phosphorylated carrier (P-HPr) donates the phosphate to the sugar with the intervention of enzyme II (Kundig, et al., 1964; Kundig and Roseman, 1966). Enzyme II appears to be a membrane-bound component which is specific for individual sugars and seems, in most cases, to be inducible.

This PEP-phosphotransferase system has since been demonstrated in Salmonella typhimurium (Simoni, et al., 1967; Levinthal and Simoni, 1969), Staphylococcus aureus (Egan and Morse, 1965a, 1965b, 1966), Aerobacter aerogenes and A. cloacae (Kundig, et al., 1964; Tanaka, et al., 1967), and Lactobacillus arabinosus (Kundig, et al., 1964), and has been shown to be involved in the accumulation of a number of sugars including galactose (Kundig, et al., 1965) and TMG (Laue and MacDonald, 1968b). Further specifics of this system will not be described since they can be found in several review articles, the most recent being those by Kaback (1970) and Lin (1970).

Koch's recent model (1971) for galactoside transport (reactions 18-30) incorporates the possibility of PEP-phosphotransferase intervention. Since several investigators have demonstrated the accumulation of phosphorylated lactose or TMG by Staph. aureus cells (Hengstenberg, et al., 1967; Kennedy and Scarborough, 1967; Laue and MacDonald, 1968a, 1968b), Koch assumes the following model to be representative of the normal mechanism. He terms E. coli the exception, citing recent genetic evidence reported by Wang, et al. (1970) as indicative of the presence of an active membrane-bound phosphatase within these cells. The function of this enzyme would be to cleave phosphate from galactosides giving the appearance, based on chromatographic evidence, of accumulation of unaltered substrate.

The upper half of Koch's model is interpreted as the mechanism for transport of all sugars, each having its specific permease, its specific enzyme II, and, in some cases, a specific phosphatase. On the outside of the membrane, substrate ($S_2$) is bound by one or more permease molecules ($P_2$) and transferred to the




transporter (T), which is defined as the substance, structure, or channel that permits diffusion of the substrate, by its interaction, through the cell membrane (Koch, 1967). Spontaneous dissociation of substrate from the transporter-substrate complex ($TS_2$) at the inner face of the membrane can occur via reaction 23; however, this mechanism is considered negligible. Therefore, for all practical purposes, Koch eliminates interference by facilitated diffusion. $TS_2$ is normally dissociated at the inner membrane interface by interaction with enzyme II and P-HPr (reaction 24), with substrate being released inside the cell as the phosphoryl derivative and free transporter reformed in the membrane. Where a phosphatase is involved, reaction 30 is carried out.

Koch (1971) does visualize additional mechanisms providing for transport. However, he dismisses the possiblity of a second energy-coupled system in tandem with the PEP system as illogical and, instead, postulates that the inner cell membrane contains a variety of mechanisms for dissociation of TS, all in parallel.

Reactions 25-29 and 21 illustrate an alternative to the PEP system although it is connected in parallel with the latter. This series of

reactions would yield nonphosphorylated substrate in the true sense of active transport. Again, specific permease molecules ($P_1$) transfer substrate ($S_1$) to the common transporter (T) forming the complex $TS_1$. Since reaction 28 is regarded as inconsequential, the dissociation of $TS_1$ must proceed through reaction 29 where energy in some form is required (~P). It is noteworthy that Koch's model predicts the absence of transport in the absence of energy.

The early transport models only speculated on the existence and function of the various components involved with permeation systems, using kinetic data. The later models, however, benefitted from information obtained from a series of investigations utilizing an osmotic shock procedure first described for enzyme release by Malamy and Horecker (1964) and Neu and Heppel (1964). The procedure involved suspending gram-negative bacteria in a solution containing sucrose, Tris buffer, and ethylenediaminetetraacetate (EDTA), centrifuging at room temperature, and resuspending in cold magnesium chloride. This procedure leaves the cells viable (able to replicate after a short lag period) even though it seems to alter the permeability barrier. The final supernatant fluid (shock fluid) was shown to contain approximately 3.5 per cent of the cellular protein including enzymes and permeases (binding proteins). There is general agreement with Heppel's proposal (1967) that the selectively released proteins are confined to a region between the cell wall and the cytoplasmic membrane.

Pardee's group (Dreyfuss and Pardee, 1965; Pardee and Prestidge, 1965; Pardee, et al., 1966) used the previously-described osmotic shock procedure, was the first to isolate and purify a binding protein from shock fluid. This protein factor, which bound inorganic

sulfate, was concluded to be a necessary component of the sulfate transport system in S. typhimurium. Using antibody interaction, Pardee and Watanabe (1968) finally proved the surface location of this sulfate binding protein.

Soon after Pardee's initial report, Piperno and Oxender (1966) showed that a nondializable factor in shock fluid from E. coli K12 binds leucine, isoleucine, and valine. Uptake of these amino acids by the cell was significantly reduced by osmotic shock. These workers compared the $K_m$ values for binding and uptake, finding them to be essentially equivalent.

More recent binder characterization studies have concentrated on specifying the interrelationship between binders and substrates. In some cases, several substrates may be transported by one permease (Penrose, et al., 1968; Anraku, 1968a; Boos, 1969), a relationship detected by competition in binding and uptake experiments, and in others, the same substrate can be accumulated via several permeases simultaneously. Rotman and co-workers have described at least four permeases which can concentrate galactose in E. coli (Rotman, 1959; Rotman and Guzman, 1961; Rotman and Radojkovic, 1964; Ganesan and Rotman, 1966; Rotman, et al., 1968), while several other groups working with this same organism have demonstrated at least two permeases which act on leucine (Furlong and Weiner, 1970; Penrose, et al., 1968; Anraku, 1968a). In Pseudomonas aeruginosa, Kay and Gronlund (1969) have reported two distinct sets of $K_m$ and $V_{max}$ values for proline transport, which may possibly indicate the presence of two permeases.

Kaback (1970) states that one of the very few ways to prove the interdependence of transport and binding is to restore transport

activity to shocked cells by addition of isolated protein factors (purified binding protein). Various researchers have tried this, obtaining results of minimal value. Under some conditions a beneficial effect is seen (Anraku, 1967, 1968c; Anraku and Heppel, 1967a, 1967b; Wilson and Holden, 1969), while under other conditions no significant effect is observed. In some instances results are extremely variable (Pardee, 1968), with nonspecific proteins producing the same effect as binding protein. Of course, osmotic shock could damage the cells in ways other than release of certain proteins. For instance, Leive (1965,a-d) has reported that treatment of cells with EDTA in the presence of Tris buffer released approximately 50 per cent of the cells' lipopolysaccharide and made them permeable to actinomycin D and other small molecules. Intracellular constituents such as the acid-soluble nucleotide pool are also released (Kaback, 1970). Because some investigators feel that this loss represents at least some of the energy reserves of the cell, they believe that this fact alone could account for loss of active transport in osmotically shocked cells. Thus, they dismiss recent studies incorporating osmotic shock treatment as inconclusive.

In a search of the literature, one finds a general lack of agreement as to what actually constitutes the energy source for active transport. According to some, it is not even connected with the previously-mentioned nucleotide pool. However, the earliest theory, one which is still quite popular, did couple ATP directly with active transport. Proponents claim that this nucleotide is involved, since uncouplers of oxidative phosphorylation such as sodium azide, 2,4-dinitrophenol, and cyanide inhibit accumulation against a gradient.

They agree with Slater (1966) in assuming that uncouplers cause the hydrolysis of high-energy intermediates that transfer energy from the electron transport chain to ATP. In addition, some workers have demonstrated the following aspects of ATP involvement: (1) Exogenous ATP specifically activates β-galactoside transport in E. coli, with many substrate molecules being transported per molecule of ATP hydrolyzed (Scarborough, et al., 1968; Carter, et al., 1968). This led Kennedy's group (Fox, et al., 1967; Scarborough, et al., 1968) to propose that ATP converts the M protein into a form with lower affinity for the substrate, causing its release inside the cell. (2) The energy supply for sodium and potassium transport by erythrocytes seems to be ATP, since this nucleotide, added to a membrane preparation, stimulates transport (Skou, 1965; Albers, 1967). (3) Uphill transport in Streptococcus faecalis is coupled to ATP production because addition of arginine stimulates galactose uptake in this organism (Wilkins, 1970).

HPr, the energy donor implicated in the phosphotransferase system, serves as a popular alternate to ATP for aiding in accumulation against a gradient. However, referring back to reactions 16 and 17, it seems more correct to specify PEP as the energy source in this instance. In any case, this energy donor is related to the bacterial transport of at least nine sugars in various organisms (Kundig, et al., 1966; Simoni, et al., 1967; Anderson, et al., 1968). The decided advantage of this group translocation system, according to Roseman (1969), is that the energy used for transport is not wasted. Instead, it is used for direct accumulation of the phosphorylated compound, yielding more net ATP than normal from glycolysis. Uncouplers of oxidative

phosphorylation may also be used to distinguish this type of energy donor: these compounds stimulate phosphotransferase transport since they increase the rate of formation of PEP (Davis and Gibson, 1967). On the other hand, iodoacetate still acts as an inhibitor (Englesberg, et al., 1961).

Three other mechanisms of energy generation have been suggested as responsible for active transport, these being proton conduction, sodium-dependent co-transport, and transport coupled to D-lactic dehydrogenase.

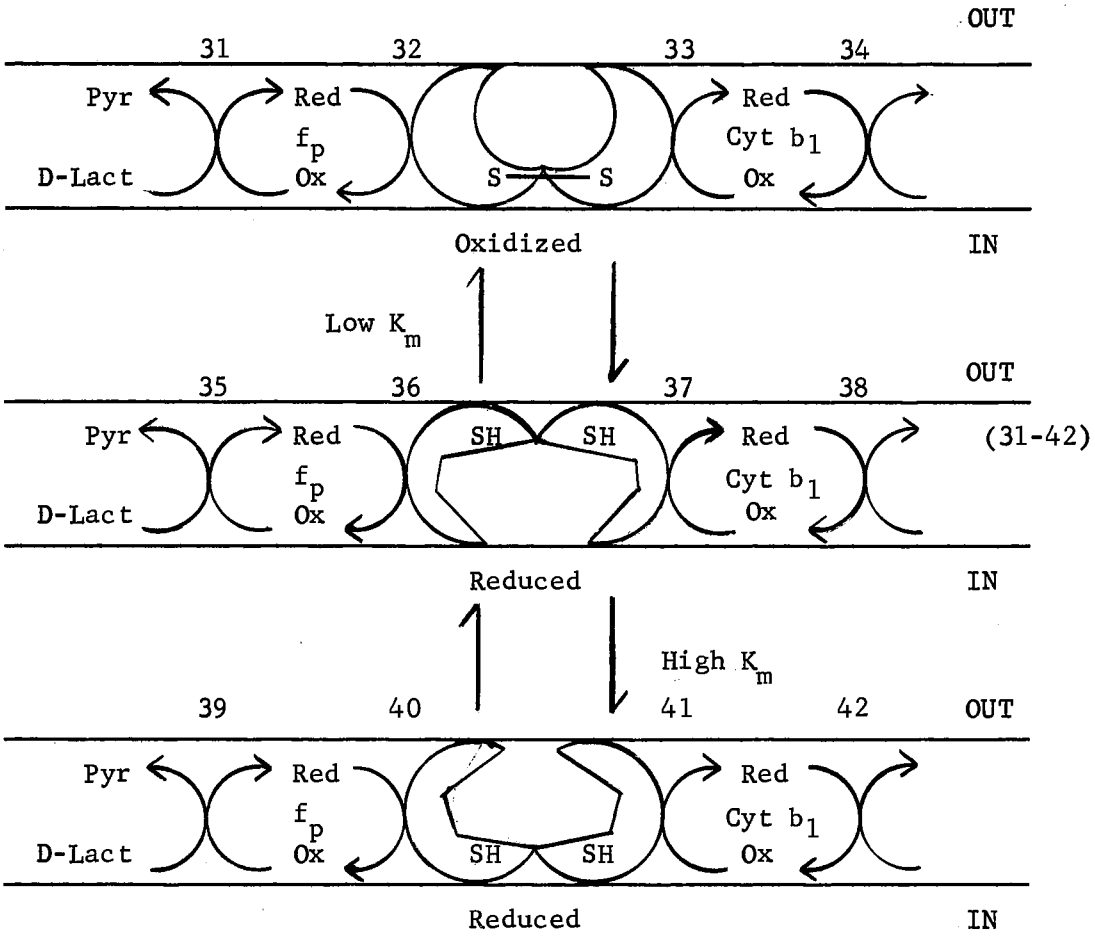
Proton conduction implies that the respiratory carriers in membranes are so arranged as to generate $H^+$ and $OH^-$ on opposite sides of the membrane. Then, by virtue of associated membrane carriers, the resulting proton gradient may be utilized directly to drive active transport (Mitchell, 1967). In the initial experiments, Mitchell's group (Mitchell, 1962; Mitchell, 1967; Mitchell and Moyle, 1967) worked with mammalian cells. Permeation of the cells to protons was measured by following equilibration of the pH after addition of a pulse of HCl. Pavlasova and Harold (1969) and Harold and Baarda (1968), using this same procedure, studied $Rb^+$, $K^+$, and $PO_4^{-3}$ transport in the fermentative organism Strep. faecalis and $\beta$-galactoside transport in anaerobically grown E. coli, with particular emphasis on the effect of uncouplers of oxidative phosphorylation. They determined that compounds such as tetrachlorosalicylanilide (TCS), carbonylcyanide-m-chlorophenylhydrazone (CCCP), tetramethyldipicrylamine (TMPA), and 2,4-dinitrophenol do not interfere with ATP metabolism yet do abolish energy coupling in active transport. In addition, they showed that these compounds actually accelerate proton translocation. Thus, inhibition of proton

conduction-driven active transport by uncouplers of oxidative phosphorylation is thought to be caused by an increased passage of protons, collapsing the gradient.

Animal cells have long been thought to transport certain sugars and amino acids by a sodium-dependent co-transport mechanism (Stein, 1967; Schultz and Curran, 1970). In other words, a substrate-specific membrane protein transports the substrate inward against the concentration gradient at the same time that sodium ions are pumped from inside the cell outward, i.e. down a concentration gradient, via a sodium pump. The net result is active transport of the substrate. Stock and Roseman (1971) have recently demonstrated this system in S. typhimurium. They have correlated it with the system previously designated TMG permease II (Prestidge and Pardee, 1965) which transports melibiose. In their studies, Stock and Roseman (1971) noted a definite dependence of the apparent $K_m$ of TMG uptake upon the $Na^+$ concentration: the stimulatory effect of the $Na^+$ caused a decrease in the $K_m$ without affecting the $V_{max}$ of entry. Also, ions such as $K^+$, $NH_4^+$, $Mg^{++}$, $Mn^{++}$, and $Ca^{++}$ showed no such effect, indicating the system to be specific.

Quite recently, D-lactic dehydrogenase has been implicated as the driving force for active transport of amino acids, $\beta$-galactosides, manganese, and potassium by membrane vesicles prepared from E. coli (Bhattacharyya, 1970; Kaback and Milner, 1970; Barnes and Kaback, 1970). In fact, there is now evidence that the carriers of these D-lactic dehydrogenase-coupled transport systems are the electron transfer intermediates themselves (Barnes and Kaback, 1971; Kaback and Barnes, 1971). Of course, the one requirement for this type of coupling is that the organism maintain some form of electron transport

system. A working model has been shown by Kaback and Barnes (1971) and is represented here by reactions 31 through 42. Only a portion of the electron transport chain is shown.




The component between reactions 32 and 33, 36 and 37, and 40 and 41 is the binding protein (permease) or membrane protein which is specific for each substrate. In the oxidized state (31-34) the carrier has a high affinity site for substrate at the outer surface of the membrane. Then, electrons coming ultimately from D-lactic acid through one or more flavoproteins reduce a critical disulfide in the carrier molecule (35-38) which results in a conformational change (39-42). This change affects the affinity of the carrier for its substrate,

bringing about release of the substrate inside the cell. After the release, the sulfhydryl form of the binder is reoxidized by cytochrome $b_1$ and electrons continue through the remainder of the chain to reduce molecular oxygen to water. Facilitated diffusion was postulated to occur via the reduced carrier through an unexplained vibrational phenomenon. The authors admit that this is only a simplified conceptual model but emphasize that it correlates with all their data.

As is evident from the previous discussion, transport systems for a number of compounds have been investigated. Uptake of all major amino acids as well as of a number of carbohydrates and intermediates in glycolysis and the Kreb's cycle have been studied in one organism or another. However, even though peculiarities in behavior of various cells in hypertonic glycerol solutions were observed as early as 1903 (Fisher), investigations on the metabolism and mechanism of uptake of glycerol were not undertaken until much later (Jacobs, et al., 1938; LeFevre, 1948; Jacobs, et al., 1950). These investigations were performed with erythrocyte membranes, with results indicating that facilitated diffusion was responsible for glycerol transport. Later, in 1965, Lin's group (Hayashi and Lin, 1965) initiated the examination of the entry of glycerol into bacterial cells. E. coli was chosen as the experimental organism. Measurement and comparison of $K_m$ values for cell growth and glycerol kinase activity showed these values to be essentially equivalent, while chromatography of a cold distilled water extract of cells exposed to $^{14}$C-glycerol for 5 minutes indicated the presence of $^{14}$C-glycerophosphate as the sole intracellular radioactive product. These results, coupled with the fact that all glycerol kinase-negative mutants selected were also transport-negative,

accounted for their conclusion that glycerol entered E. coli cells by free diffusion and was only retained inside by being metabolized to L-α-glycerophosphate. In 1968, Lin's group developed a rapid optical method which was supposed to distinguish between mediated transport and free diffusion (Sanno, et al., 1968). Upon its application to cultures of E. coli grown and induced in various ways, they determined entry of glycerol to be inducible by L-α-glycerophosphate and mediated by some component which has never been isolated or further described. Nevertheless, they then concluded that a process of facilitated diffusion serves to transport glycerol into E. coli.

Several of the pleiotropic PEP-phosphotransferase mutants of E. coli (Enzyme $I^-$ and $HPr^-$) have also exhibited inability to grow on glycerol. Although this seems to implicate the transfer of glycerol by the PEP system, several researchers have refuted this possibility (Zwaig and Lin, 1966; Berman, et al., 1970; Berman and Lin, 1971). They have shown that this particular anomaly can be specifically suppressed in various ways. The result is a revertant which grows on glycerol but still cannot grow on the other carbohydrates.

Transport of glycerol by the aerobic organism, P. aeruginosa, has been investigated by Tsay (1971). Some of this data was collected by use of the previously-mentioned rapid optical method of Sanno, et al. (1968), results of which are difficult to interpret in P. aeruginosa. This technique, used with the addition of uncouplers of oxidative phosphorylation, provided data suggesting that facilitated diffusion serves to transport glycerol into this organism. In support of this conclusion, a glycerol binding factor was released by an osmotic shock procedure and shown to be heat-labile and NEM-sensitive. However,

kinetic data suggested the presence of at least two of these binding proteins.

The present investigation was undertaken in order to extend the transport studies of Tsay, with emphasis being placed on characterization of both binding and transport of glycerol by P. aeruginosa. All transport data were collected via $^{14}C$-glycerol uptake experiments. In this manner, the possibility of energy coupling was re-examined. Two newly-selected glycerol-negative mutants (GA-73 and AH-8) were characterized and compared with PA-1-623 (the transport-negative strain used by Tsay). Binding proteins for glycerol and glucose were partially purified and characterized by means of competition experiments and an optimum pH technique. In addition, glycerol binding activity was demonstrated to be distinct from either glycerol kinase or L-α-glycerophosphate dehydrogenase activity.

In this investigation, the term free diffusion will be used to describe a non-specific transfer of solute in which neither the membrane nor any component thereof acts in a catalytic manner to expedite the process. Facilitated diffusion will indicate the involvement of some carrier which catalyzes the transfer process and increases the rate as compared with free diffusion. Active transport is similar to facilitated diffusion with the exception that solute is transferred against a concentration gradient, a process requiring an energy source. Group translocation will refer to transport via the PEP-phosphotransferase system. The terms transport, accumulation, and uptake will be used interchangeably to indicate entry of solute into the cell, as will the terms permease, binding protein, binder, and carrier, to distinguish that component which catalyzes the transfer.

Since the study of any transport system requires a knowledge of that substrate's metabolism, various known pathways for glycerol catabolism have been reviewed and are illustrated in Figure 1. Possible entry mechanisms are represented by reactions 1-3. Reaction 2 has been implicated as the process utilized in E. coli (Lin, et al., 1962; Hayashi, et al., 1964). Two pathways for the dissimilation of glycerol are known to exist in both Strep. faecalis and A. aerogenes (Jacobs and VanDemark, 1960; Lin, et al., 1960), these being represented by reactions 4, 6, and 9, and 5, 7, and 9. The latter series is supposed to function anaerobically, while the former is aerobic. The initial reactions of glycerol metabolism in P. aeruginosa were studied by Cowen (1968) who demonstrated the presence of enzymes catalyzing reactions 4, 6, and 9. The remaining series of reactions interrelating glycerol metabolism and glucose metabolism has been proposed by Heath (1971), who suggested that catabolism of glycerol by P. aeruginosa proceeds through the reaction sequence 9, 10, 11, 12, 28, 20, 21 to reach pyruvate, unless $NAD^+$-linked GAP dehydrogenase (reaction 22) is constitutive.

Figure 1. Reactions Involved in Metabolism of Glycerol and Glucose.

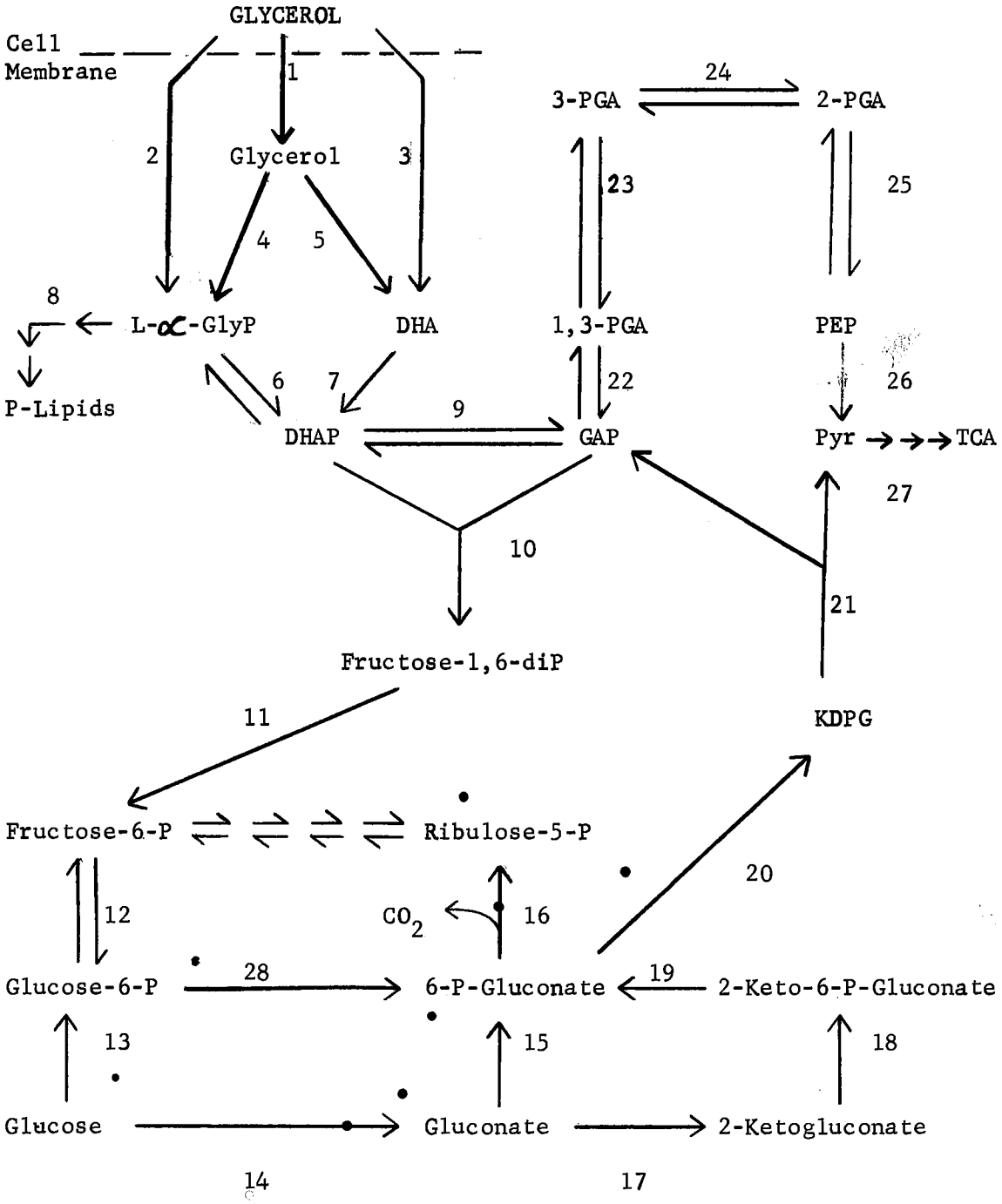
Enzymes and abbreviations are listed below. Enzyme numbers correspond to numbered reactions shown in the figure.

Abbreviations:

L-$\alpha$-GlyP, L-$\alpha$-glycerophosphate
DHA and DHAP, dihydroxyacetone and dihydroxyacetone phosphate
GAP, glyceraldehyde-3-phosphate
PGA, phosphoglyceric acid
PEP, phosphoenolpyruvate
Pyr, pyruvate
KDPG, 2-keto-3-deoxy-6-phosphogluconate

Enzymes:

1. Glycerol permease
2,4. Glycerol kinase
3,5. Glycerol dehydrogenase
6. L-$\alpha$-Glycerophosphate dehydrogenase
7. Dihydroxyacetone kinase
8. Series of reactions leading to phospholipid synthesis
9. Triose phosphate isomerase
10. Fructose diphosphate aldolase
11. Fructose diphosphatase
12. Phosphohexose isomerase
13. Glucokinase
14. Glucose dehydrogenase
15. Gluconokinase
16. 6-Phosphogluconate dehydrogenase
17. Gluconate dehydrogenase
18. 2-Ketogluconokinase
19. 2-Keto-6-phosphogluconate reductase
20. 6-Phosphogluconate dehydrase
21. KDPG aldolase
22. Glyceraldehyde-3-phosphate dehydrogenase
23. Phosphoglycerate kinase
24. Phosphoglycerate mutase
25. Enolase
26. Pyruvate kinase
27. Series of reactions leading to the Kreb's cycle
28. Glucose-6-phosphate dehydrogenase


GLYCEROL
Cell Membrane
1
2
Glycerol
3
4
5
8
L-α-GlyP
DHA
P-Lipids
6
7
9
DHAP
GAP
10
Fructose-1,6-diP
11
Fructose-6-P
Ribulose-5-P
12
Glucose-6-P
28
6-P-Gluconate
19
2-Keto-6-P-Gluconate
13
15
18
Glucose
Gluconate
2-Ketogluconate
14
17
3-PGA
24
2-PGA
23
25
1,3-PGA
PEP
22
26
Pyr → → → TCA
27
21
KDPG
20
CO2
16

# CHAPTER II

# MATERIALS AND METHODS

## Strains of Bacteria

Two varieties of the wild type strain of Pseudomonas aeruginosa (designated PA-$1_L$ and PA-$1_S$) were used in this study. The original PA-1 was obtained from B. W. Holloway of the University of Melbourne. This strain displays the normal appearance and metabolic characteristics of P. aeruginosa as listed in Bergey's Manual (Breed, et al., 1957) with a colonial morphology on glucose minimal medium A as follows: circular, convex, entire, butyrous, opaque and greenish yellow. In 0.5 per cent glycerol minimal liquid medium A with aeration, it reaches 20 per cent transmittance with even turbidity in approximately 12 hours.

The small colony variant (PA-$1_S$) was spontaneously derived from PA-$1_L$ and is metabolically similar to PA-$1_L$ with regard to the reactions of interest in this study except that it grows more slowly in liquid culture (which is consistent with its smaller size on plates). In all liquid cultures it forms flakes, producing a grainy-appearing turbidity. In 0.5 per cent glycerol minimal medium A, it requires approximately 27 to 30 hours to reach a per cent transmittance of 20. On glucose plates the colonial morphology differs from that of PA-$1_L$ only in that PA-$1_S$ colonies are punctiform, slightly raised, and dry.

Glycerol-negative mutants used in this study were derived from $PA\text{-}1_L$ by nitrosoguanidine treatment. These include: GA-73, AH-8, obtained from Floyd E. White, Jr., and PA-1-623, obtained from Clint Cowen (1968).

## Media

Minimal medium A was a modification of Robert's M-9 medium (Roberts, et al., 1957) and contained the following constituents per liter: $Na_2HPO_4 \cdot 7H_2O$, 8.2 g; $KH_2PO_4$, 2.7 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $NH_4Cl$, 1.0 g; $FeSO_4$, 0.1 per cent solution, 0.5 ml; distilled water to volume. The $MgSO_4 \cdot 7H_2O$ was autoclaved separately as a 40 per cent solution (w/v). The carbon sources, sodium lactate, glucose, and glycerol, were autoclaved separately as 20 per cent (w/v) solutions, while all other carbon sources, also at this concentration, were filter-sterilized through 47 mm Gelman filters, H. A. 45 $\mu$. All substrates were added aseptically to a final concentration of 0.5 per cent (w/v) to minimal medium A which had been autoclaved in the flasks to be used for growth of the organism.

The term minimal medium B is used to designate a medium identical to minimal medium A except that $MgSO_4 \cdot 7H_2O$ and a carbon source were omitted. It was used for washing cells in most experiments and for suspension of cells during transport experiments.

## Plates

All minimal medium plates were prepared by autoclaving the previously mentioned amounts of $Na_2HPO_4 \cdot 7H_2O$, $KH_2PO_4$, and $NH_4Cl$ in one flask, 0.5 per cent (w/total volume) carbon source in a second flask,

and two per cent (w/total volume) Bacto-agar (Difco) plus the required amounts of $MgSO_4 \cdot 7H_2O$ and $FeSO_4$ in a third flask. All flasks and contents were autoclaved at 250 C and 15 psi for 15 minutes. After sterilization the flasks were cooled to 47 C in a preheated water bath and all constituents were added aseptically to the large flask containing Bacto-agar, $MgSO_4 \cdot 7H_2O$ and $FeSO_4$ and mixed well before being distributed into plates.

Nutrient agar was rehydrated according to directions on the bottle, autoclaved under the same conditions as the minimal media, cooled to 47 C, mixed and poured into plates.

Sterile syringe pipettes (A. H. Thomas Co.) were used to deliver 25 ml of agar medium per sterile plate. After solidification, the plates were inverted, allowed to dry approximately 48 hr at room temperature and then stored at 0-5 C. Plates to be spread with cells were dried an additional 24 hr at 37 C before use.

## Cultivation of Bacteria

All bacterial strains were streaked on nutrient agar slants, allowed to grow 18-24 hr at 37 C, and placed at 0-5 C for storage. Glycerol-negative strains, PA-1-623, AH-8, and GA-73 were maintained more efficiently by placing 1 ml of 20 per cent sterile glycerol on the pre-grown slant before storage at 0 C.

Minimal medium A plus the desired carbon source was the growth medium of choice. For the majority of experiments, cells were grown in flasks placed on a reciprocal shaker at 37 C. Growth continued until the suspension attained a reading of 20 per cent transmittance as measured in an 18 mm tube at 540 nm on a Coleman Junior Spectrophotometer.

Cells were harvested by centrifugation at room temperature when physiologically active cells were desired, or at 0 C otherwise, and washed twice with minimal medium B unless stated otherwise. When induction was required, once-washed cells were resuspended to the original volume in minimal medium A plus the desired carbon source at 0.5 per cent and placed on a reciprocal shaker at 37 C for four hours. Before use for experimentation, the induced cells were harvested and washed twice with minimal medium B.

Whenever mutants were used in an experiment, a 0.05 ml sample was removed from each growth flask, inoculated into 6.0 ml of 0.5 per cent glycerol minimal medium A and incubated with aeration at 37 C to check for reversion.

## Chemicals

Carbenicillin was a gift from Beecham Pharmaceuticals. D-cycloserine was a product of Mann Research Laboratories. Absolute ethanol was produced by U. S. Industrial Chemicals. Aquasol was a product of New England Nuclear. Adonitol was obtained from Difco Laboratories. Products of Aldrich Chemical Company were N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and 1,3-propanediol. Sodium pyruvate and 3(4,5 dimethyl thiazolyl 1-2)2,5 diphenyl tetrazolium bromide (MTT) were obtained from Nutritional Biochemicals. Glycerol-U-$^{14}$C and the scintillation fluors were purchased from Amersham/Searle. Products of Eastman Kodak Company were N-ethylmaleimide, 2,4-dinitrophenol, dulcitol, hydrazine, and 1,2-propanediol.

Chemicals obtained from Fisher Scientific Company (Certified A.C.S. grade) included the following: sodium chloride, sucrose,

dextrose, 85% phosphoric acid, potassium cyanide, glycerin, sodium azide, tertiary potassium phosphate, sodium arsenate, toluene, absolute methanol, potassium hydroxide pellets, phenol reagent, magnesium chloride, D-fructose, sodium citrate, and mannitol. Sodium lactate was obtained from Fisher as a 60% syrup.

Chemicals from Calbiochem (A grade) were as follows: histidine (hydrochloride, hydrate), diphosphopyridine nucleotide ($NAD^+$), tris-(hydroxymethyl)aminomethane (Tris), N,N Bis(2-hydroxyethyl)glycine (Bicine), adenosine-5'-triphosphate (ATP), dihydroxyacetone dimer, L-methionine, succinic acid (disodium salt, hexahydrate), and uniformly labelled $^{14}C$-glucose.

The following chemicals were purchased from the J. T. Baker Company (Analyzed Reagent grade): disodium (ethylenedinitrilo) tetraacetate·$2H_2O$ (EDTA), potassium phosphate (monobasic), potassium phosphate (dibasic), ammonium sulfate, sodium carbonate (anhydrous powder), cupric sulfate (pentahydrate), potassium sodium tartrate, and n-propanol.

Those chemicals produced by Sigma Chemical Company include: bovine serum albumin, DL-glyceric acid (calcium salt, dihydrate), D-gluconic acid (potassium salt), $\beta$-glycerophosphate (disodium salt, pentahydrate), oligomycin, antimycin A, chloramphenicol, meso-erythritol, DL-$\alpha$-glycerophosphate (disodium salt, hexahydrate), and L-$\alpha$-glycerophosphate dehydrogenase (rabbit muscle).

## Protein Determination

The protein assay of Sutherland et al. (1949) was used to determine the protein content of shock fluids, partially purified binding

protein solution, and cell-free extracts. Standards (bovine serum albumin) and samples containing 30 to 90 μg/ml of protein were diluted to 1.0 ml with distilled water. Five milliliters of a fresh solution containing 1.0 ml of 4.0 per cent potassium sodium tartrate and 1.0 ml of 2.0 per cent $CuSO_4 \cdot 5H_2O$ per 100 ml of 4 per cent $Na_2CO_3$ were added to each sample and to a water blank and mixed. After 40 minutes incubation at room temperature, 0.5 ml of a freshly prepared 0.67 N phenol reagent solution was added to each tube and mixed immediately. The optical density was determined at 660 nm after 15 minutes.

Recently a report by Gregory and Sajdera (1970) noted that certain buffers interfere with color formation in protein determinations utilizing the Folin-Ciocalteau method. Standards consisting of 30, 60, and 90 μg/ml bovine serum albumin were made up in bicine buffer, phosphate buffer, and sucrose-tris-EDTA solution in the same proportions as in samples. In all cases, color production was increased. Therefore, standards made up in the appropriate solutions were run simultaneously with each set of samples.

## Preparation of Cell-Free Extracts

The appropriate strain of P. aeruginosa was grown (in a volume of at least 200 ml) and induced, when necessary, according to the procedure described previously. Cells were harvested by centrifugation at 0 C and washed twice with 0.85 per cent (w/v) saline. The final pellet was frozen at -20 C for not longer than one week.

Frozen pellets were thawed by resuspending in cold 0.1 M bicine, pH 8.5, to a volume equivalent to approximately 2 per cent of the original growth volume. These cells were broken by sonication with a

Bronson sonifier, being subjected to 15 second bursts and intermittent cooling in ice until a clear suspension was obtained. Calbiochem MiniBeakers (5, 10, and 25 ml size) were found ideal for efficient breakage. The clear suspension was centrifuged at 0 C and 20,200 x g for 10 minutes in a refrigerated centrifuge to obtain the crude cell-free extract (supernatant), which was placed on ice and assayed immediately. A 1:20 dilution of the cell-free extract was made and a 0.1 ml sample was frozen for a later protein determination.

## Enzyme Assays

Undialyzed crude cell-free extracts were assayed spectrophotometrically for glycerol kinase (ATP:glycerol phosphotransferase, EC 2.7.1.30) and L-α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate [acceptor] oxidoreductase, EC 1.1.99) activities on a Cary 14 dual beam recording spectrophotometer. Total volume in the blank and sample cuvettes was 3.0 ml with the contents of the two differing only in the substitution of glass-distilled water for substrate in the blank. For both assays, constituents were added in the order mentioned below with the cuvettes being mixed by inversion after the addition of substrate. The assays were performed at room temperature. Appropriate controls were run to insure the purity of all enzymes and reagents added to the reaction cuvette. However, it was noted that the kinase reagents displayed a very slight activity ($\Delta$O.D./min=0.002) which was subtracted from each rate before calculation of specific activity. A unit of enzyme (U) was defined as that amount required to convert one micromole of substrate to product(s) per minute under the specified conditions.

Glycerol Kinase

The method of Lin et al. (1962) was adapted for use with P. aeruginosa extracts. An amount of crude cell-free extract varying between 0.2 ml and 0.5 ml was preincubated at least 12 minutes (in both cuvettes) with 0.1 ml of 0.05 M ATP. The remaining reaction mixture consisted of: 0.2 ml of 0.02 M $NAD^+$; approximately 5 U of rabbit muscle L-α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: $NAD^+$ oxidoreductase, EC 1.1.1.8); 0.1 M Tris buffer, pH 7.5, (0.9 to 1.2 ml depending upon the amount of extract); 0.3 ml of 3 N hydrazine; 0.6 ml of 0.1 M $MgCl_2$; and 0.3 ml of 0.1 M glycerol. Formation of NADH was followed at 340 nm. The millimolar extinction coefficient used to calculate the specific activity was 6.2 (Dawson et al., 1959).

L-α-Glycerophosphate Dehydrogenase

This assay was also a modification of that of Lin et al. (1962). The reaction mixture consisted of the following: 2.1 to 2.4 ml (depending on amount of extract) of 0.1 M Tris buffer, pH 7.5; 0.2 ml of 0.15 M KCN; 0.2 to 0.5 ml of cell-free extract; 0.1 ml of 1 mg/ml MTT; and 0.1 ml of 1.0 M DL-α-glycerophosphate. Enzyme activity was detected by spectrophotometric measurement, at 550 nm, of the rate of reduction of the tetrazolium dye, MTT, to its formazan. The millimolar extinction coefficient used for calculation of specific activity was 9.9 (Sowerby and Ottaway, 1966).

## Transport of Glycerol-U-$^{14}C$

Several types of transport experiments were run. These varied only in the additions of "cold" substrate or inhibitor and in the time of

sampling. In each case, the desired strain of P. aeruginosa was grown and treated as described previously. After the last centrifugation, the pellet was resuspended in minimal medium B to a per cent transmittance of 45. A 0.1 ml volume of $10^{-6}$ dilution of this cell suspension was spread onto each of three glucose minimal medium plates, and after 24 hr of incubation at 37 C the colonies were counted and the average count was used to calculate concentration of viable cells. Either 24.9 ml of the cell suspension were placed into a 250 ml Erlenmeyer flask or 12.45 ml were placed into a 125 ml Erlenmeyer flask, depending upon the number of samples to be taken. The flask was then incubated for 10 minutes at 37 C and 134 oscillations per minute in a shaking water bath. When required, "cold" substrate additions to 0.01 M were made at one minute before zero time and inhibitor additions at their appropriate concentrations were either made at 30 minutes before zero time or during uptake. In each case, untreated controls were pre-incubated for the same period of time under identical conditions except for the addition of substrate or inhibitor.

At zero time, 15.9 nmoles of glycerol-U-$^{14}$C (14.3 mCi/mM) in a volume of 0.1 ml were added to the 24.9 ml volume of cell suspension or 0.05 ml of solution containing 7.95 nmoles glycerol-U-$^{14}$C was added to the 12.45 ml volume. This yielded a concentration of approximately 0.6 nM/ml of glycerol-U-$^{14}$C. At the time intervals appropriate for each experiment, 1.0 ml samples were removed with a 1.0 ml automatic syringe and filtered immediately through a pre-wetted Gelman Metricel filter (47 mm diameter, 0.45 $\mu$ pore size) placed on a metal screen in a Millipore filter funnel apparatus. The vacuum was opened to each filter tower 25 seconds before sampling, and filters were washed with

1.0 ml of minimal medium B at 60 seconds and at 105 seconds after sampling. After all samples had been taken and all washings completed, filters were removed, placed in scintillation vials and dried with hot air. They were then crushed in order to form a transparent suspension upon addition of 10 ml of scintillation fluid B. As a control, 0.01 ml of glycerol-U-$^{14}C$ was placed in a separate vial in 10 ml of scintillation fluid B. Thirty minutes after the addition of scintillation fluid, the vials were shaken vigorously to emulsify the filter particles. The vials then remained at least four hours at room temperature without being disturbed before being counted on a Nuclear Chicago Model 720 scintillation counter. Counts were corrected for quenching, and uptakes were plotted as nanomoles of glycerol-U-$^{14}C$ taken up per milligram dry weight of cells, using the viable cell counts and assuming the dry weight of one cell to be $2 \times 10^{-13}$ g (Luria, 1960).

## Measurement of $^{14}CO_2$ Accumulation During Transport

Cells grown or induced as described previously were harvested by centrifugation at room temperature, washed twice with minimal medium B and resuspended to 45 per cent transmittance in minimal medium B. The cell suspension was plated to determine viable count as in transport experiments. A 2.5 ml volume of cell suspension was then placed into each of the 25 ml Erlenmeyer flasks used in the experiment. Into a small Nalgene vial cap, suspended in each flask by wire (Figure 2), was placed 0.1 ml of 20 per cent KOH. The flask was then sealed with a serum stopper. Individual flasks were preincubated ten minutes at 37 C and 134 oscillations per minute in a shaking water bath when a "cold"

substrate was added and 11 minutes under the same conditions when control experiments were run. The final concentration of "cold" substrate in the flask was 0.01 M.

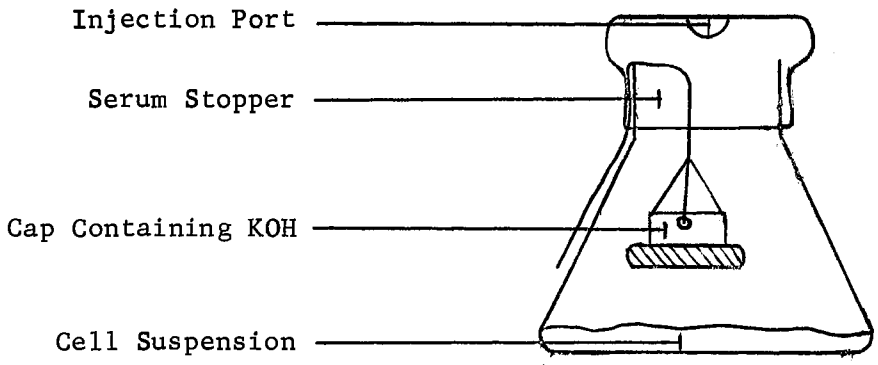



Figure 2. Flask Used for Measuring $^{14}CO_2$ Accumulation

When inhibitors were used, the preparation procedure was altered so that the required concentration of inhibitor was added to the cell suspension before it was placed in the flask. After following the remaining preparation procedure, the flasks were preincubated under the aforementioned conditions for 30 minutes.

In all cases, 0.01 ml of 159 μM glycerol-U-$^{14}C$ was injected into each flask with a 50 μl syringe at zero time. Care was taken so that the syringe tip was below the KOH-cap but slightly above the level of the cell suspension. After the appropriate incubation time for each flask, the reaction was stopped by injecting 1.0 ml of 5 N HCl with a 1.0 ml automatic syringe, taking care not to release the plunger and remove $CO_2$. The acid also served to release any $^{14}CO_2$ dissolved in the medium. The flasks were allowed to shake for an additional thirty minutes, after which they were removed from the shaking water bath and

unstoppered. The entire cap and contents were carefully transferred to a scintillation vial. Immediately, 18 ml of scintillation fluid C were added to the vial. Inversion of the capped vial several times provided sufficient mixing. Samples were counted with correction for quenching as indicated in previous experiments. As a control, 0.01 ml of glycerol-U-$^{14}C$ was placed in a vial with 18 ml of the same scintillation fluid and counted. Results are plotted as dpm x $10^3$/mg dry wt of cells.

## Shock Procedure

The various strains to be shocked were grown, and when necessary induced, according to the procedure described previously. The cells were harvested by centrifugation at 10,000 x g at room temperature and were washed twice with 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 7.0. For shocking, the washed pellet was resuspended in a cold solution containing either 0.5 M sucrose-0.05 M Tris-0.001 M disodium (ethylenedinitrilo)tetraacetate (EDTA) or 0.5 M sucrose-0.05 M Tris-1 mg/ml disodium EDTA, depending upon the experiment. The amount of solution (5 to 45 ml) was dependent upon the size of the pellet and the amount of shocking desired. This suspension was placed at room temperature and was periodically mixed by aspirating with a pipette. After at least thirty minutes, a 0.1 ml sample was removed, pipetting 0.05 ml into 10 ml of 0.05 M sucrose and the remaining 0.05 ml into 10 ml of distilled water in order to confirm the presence of fragile rods. A well-shocked sample displayed a loss of optical density of at least 15 per cent during a fifteen minute interval in distilled water.

The shock suspension was then centrifuged at 0 C for fifteen minutes, and the supernatant was harvested as the shock fluid. The pellet was saved and, where necessary, was assayed for kinase and dehydrogenase activity. One tenth milliliter of the shock fluid was immediately removed for the protein determination. The rest, in most cases, was used immediately for further experimentation since the binding activity of PA-1 was unstable.

## Equilibrium Dialysis

Equilibrium dialysis cells (Chemical Rubber Co.) were prepared for use by washing with soapy water and rinsing with tap and distilled water followed by complete drying. A six-inch segment of dialysis tubing (1 1/8 inch inflated diameter) was cut along the edges and separated. These pieces were then either boiled in 0.001 M EDTA (no pH adjustment) or 0.3 M sucrose-0.001 M disodium EDTA, pH 8.0, depending on the experiment. The single-thickness boiled membrane was rinsed with triple distilled water and placed onto one-half of the equilibrium dialysis apparatus. The other half was then aligned and bolted into place.

In all cases, 1.0 ml of shock fluid or partially purified binding protein solution was added to one side of a cell in the equilibrium dialysis apparatus with a 1.0 ml syringe. To the other side of the membrane was added a 1.0 ml volume which contained either 0.69 $\mu$M glycerol-U-$^{14}$C (14.3 mCi/mmole) or 0.16 $\mu$M glucose-U-$^{14}$C (60 mCi/mmole) made up in glass-distilled water. Although equilibration and binding took place in seven hours, the cells were usually sampled after twelve hours of incubation on an equilibrium dialysis shaker.

Sampling consisted of removing 0.1 ml of solution from each side of the dialysis membrane and placing it into a scintillation vial. Immediately, 10 ml of scintillation fluid A were added to each vial after which the samples were counted with correction for quenching.

Binding in equilibrium dialysis experiments was expressed as a ratio, R, which equaled the dpm in a 0.1 ml aliquot taken from the protein solution divided by the dpm in a 0.1 ml aliquot taken from the radioactive substrate solution. It is noted that when $R \leq 1.0$ there is no apparent binding activity.

## Isolation of Binding Protein

Cells were grown to approximately 25 per cent transmittance in two liters of the specified medium. They were harvested in centrifuge bottles at 10,000 x g at room temperature, washed twice with 0.05 M Tris, pH 7.0, and shocked with 30 ml of 0.5 M sucrose-0.05 M Tris-0.001 M disodium EDTA according to the shock procedure described previously.

One milliliter of the crude shock fluid was immediately placed in an equilibrium dialysis cell to verify the initial presence of glycerol binding. In some cases a second 1.0 ml sample was checked for glucose binding activity.

The remaining shock fluid was placed in a beaker in an ice bath. The solution was mixed with a magnetic stirrer (E. H. Sargent) at its lowest setting while solid $(NH_4)_2SO_4$ was added. Samples were taken at intervals of 10 per cent saturation between 50 and 80 per cent.

To test the precipitate for binding activity, the suspension was centrifuged at 0 C and 20,200 x g for fifteen minutes, and the pellet (partially purified binding protein) was resuspended in a proportionate

amount of distilled water or buffer (as specified in each experiment). Two tenths milliliter of this partially purified binding protein solution (PPBPS) was removed for a protein determination. One milliliter aliquots of the remaining solution were dialysed against labelled glycerol and glucose.

## Determination of pH Profile of Isolated Binding Protein

Cells were grown, harvested, and shocked according to the procedures previously described. The shock fluid was treated as described previously to the point of its final centrifugation. The terminal treatment consisted of placing equal amounts of 70 per cent saturated ammonium sulfate-shock fluid suspension into seven centrifuge tubes and centrifuging in the cold at 20,200 x g for 15 minutes. The supernatant was discarded and the tubes were dried completely with cotton swabs. Phosphate buffers at 0.05 M and pH's of 4, 5, 6, 7, 8, 9, and 10 were prepared utilizing $H_3PO_4$, $KH_2PO_4$, $K_2HPO_4$, and $K_3PO_4$. Three milliliters of the specified buffer were placed in each centrifuge tube. The tubes were kept on ice while the precipitate dissolved and until being placed in equilibrium dialysis cells.

Equilibrium dialysis tubing was boiled in 0.001 M EDTA and rinsed with glass distilled water. Two 1.0 ml samples were removed from each centrifuge tube; 1.0 ml of PPBPS was dialyzed against glycerol-U-$^{14}C$ and 1.0 ml against glucose-U-$^{14}C$. A 0.2 ml sample of PPBPS was removed from each tube for a protein determination. Curves displaying the pH profile for a given strain on a specified growth medium were plotted as R vs. pH.

## Assay of Binding Competition

Binding competition studies were carried out on crude shock fluids and on PPBPS from wild-type cells. Normal cultivation, harvest, and shock procedures were followed. For the case of the PPBPS, the protein isolation procedure described above was followed. The pH was adjusted to 5.0 and 7.0 for different samples when checks were made to determine binding competition with glucose-U-$^{14}$C and glycerol-U-$^{14}$C, respectively. After the partially purified binding protein had been dissolved in buffer, the remaining procedure was identical to the following one for shock fluids.

Each competitor was placed into an individual tube to produce a final concentration of 0.01 M upon addition of 1.5 ml of shock fluid. When inhibitors were tested, the format differed only as to the concentration used in the specific experiment. The shock fluid-competitor solution remained at room temperature at least 10 minutes, with intermittent mixing. Individually, a 1.0 ml aliquot was removed from each tube and placed into a separate equilibrium dialysis cell. An untreated control was added to the last cell, after which the appropriate radioactive substrates were added to their respective compartments. The remaining procedure was that described for equilibrium dialysis using dialysis tubing boiled in 0.5 M sucrose-0.001 M EDTA solution adjusted to pH 8.0 for shock fluids and in 0.001 M EDTA for PPBPS.

Inhibition of binding by competitors or inhibitors was calculated by the following equation:

$$\% \text{ inhibition} = 100 - \left(\frac{R \text{ sample} - 1.00}{R \text{ control} - 1.00}\right) \times 100$$

## Scintillation Fluids

It was noted early in this study that addition of 47 mm membrane filters to scintillation fluid in vials reduced counting efficiency, as did addition of a basic solution such as KOH. Therefore, all scintillation counting was optimized between 40 and 60 per cent efficiency with the utilization of three scintillation fluids.

Scintillation fluid A consisted of absolute ethanol-toluene 4:6 (v/v), 4 g/l of 2,3-diphenoloxazole (PPO) and 200 mg/l of 1,4-bis [2-(5-phenyloxazolyl)benzene] or POPOP. Scintillation fluid B was a 1:1 mixture of scintillation fluid A and commercially obtained Aquasol. Scintillation fluid C or Turner's scintillation fluid (Chabner and Livingston, 1970) consisted of absolute methanol-toluene 3:7 (v/v) with 3 g/l PPO and 100 mg/l of POPOP.

# CHAPTER III

## EXPERIMENTAL RESULTS

### Transport and Binding of Glycerol-U-$^{14}$C by Wild-Type Variants

Since PA-$1_L$ (large colony variant) and PA-$1_S$ (small colony variant) were used interchangeably in all types of experiments in this study, it was necessary to compare transport and binding data for the two strains. Figure 3a illustrates the results obtained when PA-$1_L$ and PA-$1_S$, grown in 0.5 per cent glycerol minimal medium A, were checked for their ability to transport glycerol-U-$^{14}$C. The curves representing glycerol uptake by the two strains appeared to be equivalent in all respects. The one-minute value of 0.73 nmoles/mg dry wt of cells was the same for each strain as was the plateau value of 1.17 nmoles/mg dry wt of cells reached at three minutes.

Part of the PA-$1_L$ cell suspension used to measure the uptake shown in Figure 3a was placed into a 125-ml Erlenmeyer flask and boiled for six minutes. After transferring 24.9 ml of this suspension to a 250-ml Erlenmeyer flask, uptake of glycerol-U-$^{14}$C was again measured. These data are also shown in Figure 3a. It was evident that heated wild-type cells neither took up nor bound (on the outer surface or at binding sites) significant amounts of radioactive glycerol.

Glycerol-grown PA-$1_L$ and PA-$1_S$ cells were shocked and the shock fluids were used in equilibrium dialysis experiments with radioactive

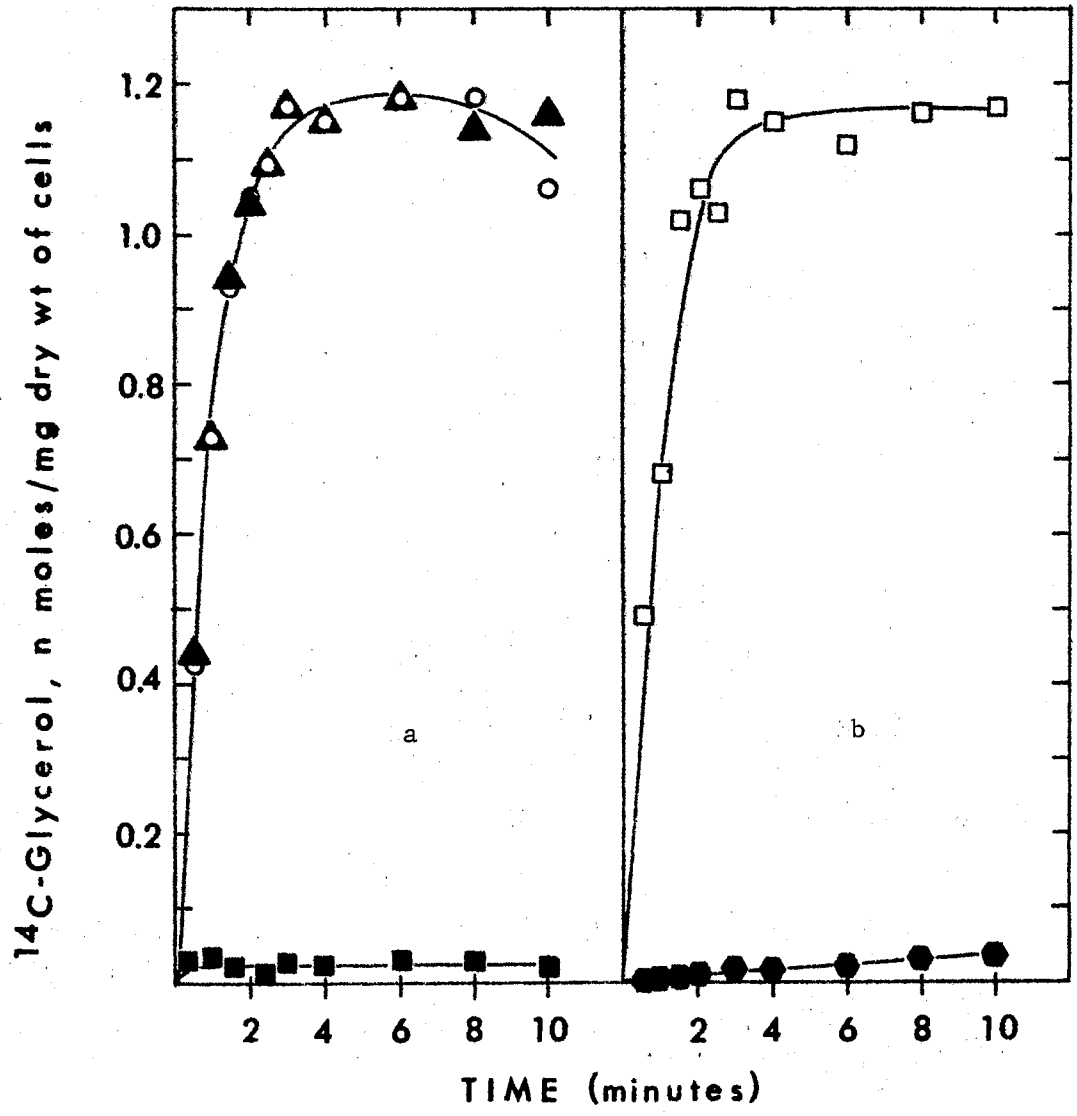
Figure 3. Transport of Glycerol-U-$^{14}$C by Glycerol-Grown PA-$1_L$ Cells, PA-$1_S$ Cells, and PA-$1_S$ Fragile Rods.

a. Untreated cells were prepared for transport assay by the procedure described in Materials and Methods. Boiled PA-$1_L$ cells were heated at 100 C for 6 min, after which transport was measured.

Glycerol-U-$^{14}$C transport by PA-$1_L$ (▲); PA-$1_S$ (o); and by boiled PA-$1_L$ (■).

b. Glycerol-grown PA-$1_S$ (whole cells) were washed and suspended to 45 per cent transmittance in 0.5 M sucrose, after which transport was measured with substitution of 0.5 M sucrose for minimal medium B. A portion of the cell suspension was shocked. The shock pellet (fragile rods) was suspended to 45 per cent transmittance in 0.5 M sucrose and also tested for transport ability.

Transport by whole cells in sucrose (□) and by fragile rods in sucrose (●).


$^{14}$C-Glycerol, n moles/mg dry wt of cells
1.2
1.0
0.8
0.6
0.4
0.2
a
b
2
4
6
8
10
2
4
6
8
10
TIME (minutes)

glycerol and glucose to determine their respective binding capacities. At the same time, bovine serum albumin at 300 μg/ml (suspended in 0.5 M Tris-0.001 M EDTA) was tested for binding of glycerol and glucose in order to define protein specificity for these substrates. Table I lists the results which indicated that the two wild-type variants bound approximately equal amounts of glycerol and glucose, respectively. Also, bovine serum albumin displayed no affinity for either substrate, at least negating the possibility that glycerol and glucose could bind nonspecifically to any protein.

TABLE I

BINDING OF GLYCEROL AND GLUCOSE BY SHOCK FLUIDS FROM PA-$1_L$ and PA-$1_S$

| Protein Solution | Binding (R) | |
|---|---|---|
| | Glycerol | Glucose |
| Shock fluid from PA-$1_L$ | 1.98 | 3.34 |
| Shock fluid from PA-$1_S$ | 1.96 | 2.23 |
| Bovine serum albumin | 0.95 | 0.98 |

A portion of the PA-$1_S$ shock pellet, retained from the binding experiment and consisting of fragile rods, was suspended in 0.5 M sucrose to 45 per cent transmittance and a sample was plated as was usually done in transport experiments. Glycerol transport activity was

measured and compared to that of a control of untreated PA-$1_S$ cells suspended in 0.5 M sucrose. It was important to determine the effect of osmotic shock upon transport and also to determine whether sucrose, at this high concentration, had some effect other than protection of fragile rods. In both instances, optical density readings were taken before and after measurement of transport and no changes were observed. Results of this experiment, shown in Figure 3b, indicated that shocked wild-type cells almost completely lost their ability to transport glycerol. The rate of glycerol uptake by fragile rods became linear and reached a maximum of only 3.4 per cent of the control at 10 minutes. It is noteworthy, also, that 0.5 M sucrose had no noticeable effect on glycerol transport by whole cells of PA-$1_S$, compared to glycerol transport by PA-$1_S$ in the usual transport medium (Figure 3a).

## Induction of Glycerol Transport Activity and Glycerol Degradative Enzymes in PA-$1_L$

Tsay (1971) had determined that, in wild-type cells, the glycerol degradative enzymes were induced after four hours of incubation in glycerol minimal medium. She also noted that glycerol transport by glucose-grown PA-1 was increased after a four-hour induction period in glycerol medium. For further experimentation in this study, it was necessary to determine the true induction times for glycerol transport and for the glycerol degradative enzymes (glycerol kinase and L-$\alpha$-glycerophosphate dehydrogenase). Therefore, 1200 ml of PA-$1_L$ cells were grown to late log phase in 0.5 per cent glucose minimal medium A, after which they were harvested at 10,000 X g at room temperature, washed with minimal medium B, and resuspended in 1600 ml of minimal

medium A containing 0.5 per cent glycerol and 0.5 per cent glucose. A 200-ml sample was removed after 30 minutes incubation, a 15-ml portion being checked for optical density and $^{14}$C-glycerol transport, while the remaining cells were prepared for assay of enzyme activities. After determining the O.D. of a 1/10 dilution of the cell suspension, this 10-ml portion was membrane-filtered and the filtrate was frozen for use in later determinations of the concentrations of glucose and glycerol. Glucose was determined by the Glucostat method of Worthington Biochemical Corporation (Cowen, 1968). Komolrit's modification (Komolrit, 1965) of the method of Neish (1952) was used to determine the glycerol content. At one hour intervals (up to and including eight hours) additional 200-ml samples were removed and treated identically.

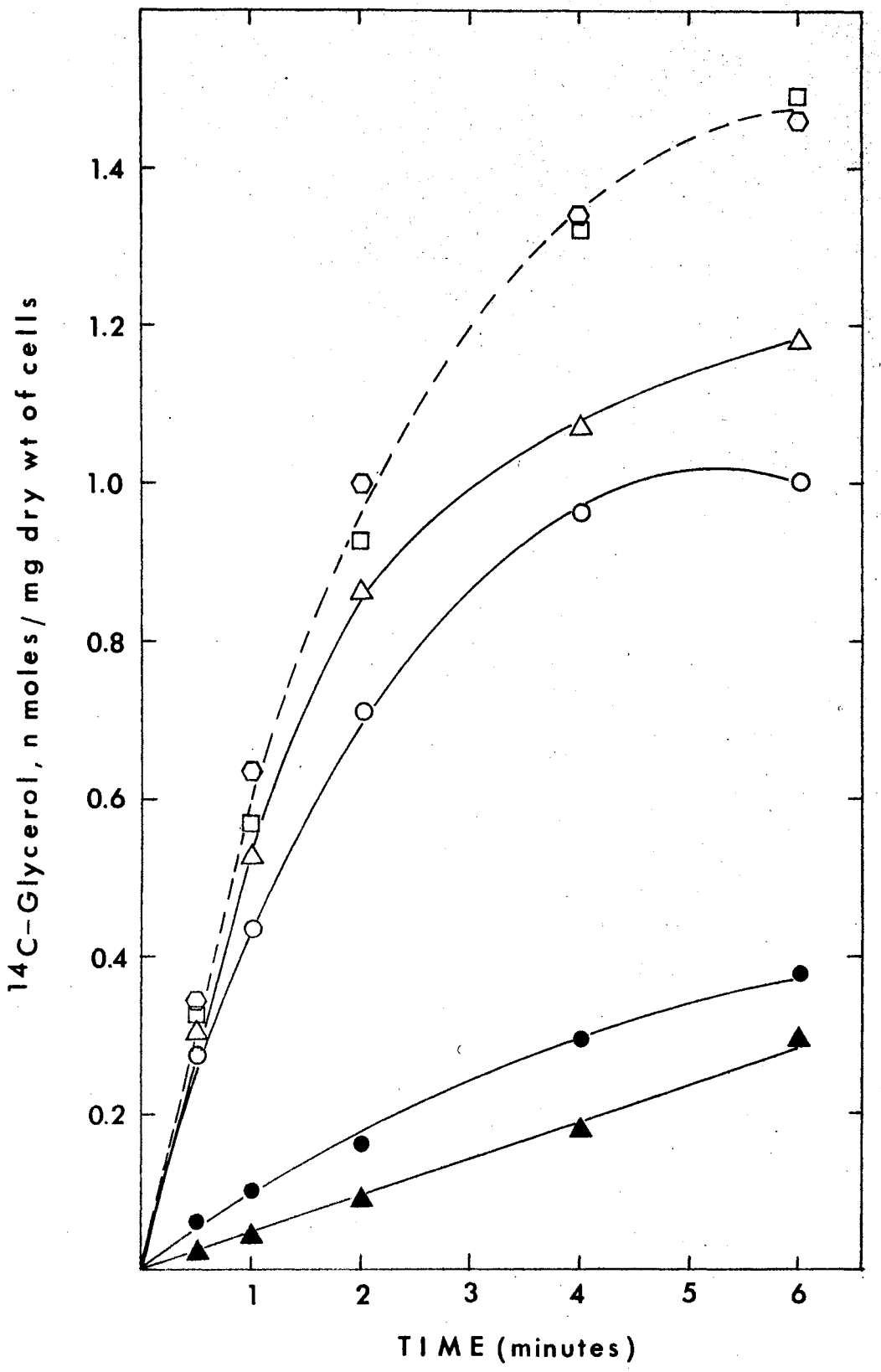
Figure 4 shows the glycerol transport curves for cells tested after 0.5, 1, 2, 3, 4, and 8 hours of glycerol induction. The intermediate curves (for cells taken at hours 5 through 7) were not included since they were essentially the same as curves representing transport by four-hour and eight-hour cells. Interestingly enough, the 30-second uptake value remained relatively constant after two hours of glycerol induction (including the values not shown), while at least four hours of induction was required to produce consistent plateau levels.

For comparison, Table II lists the optical densities of the cell suspensions (after a 1/10 dilution) and the corresponding specific enzyme activities of the respective samples. Disappearance of both substrates occurred simultaneously, with the majority being removed between one and three hours. Three to four hours of induction with glycerol seemed necessary to produce constant enzyme activities.

Figure 4. Induction Pattern for Glycerol-U-$^{14}$C Transport by PA-$1_L$

Wild-type cells were grown in 0.5 per cent glucose minimal medium A, washed, and induced for various periods of time in minimal medium A containing both 0.5 per cent glycerol and 0.5 per cent glucose. The initial sample was removed 30 minutes after resuspension of cells in the induction medium. Additional samples were removed at one-hour intervals up to, and including, eight hours.

Glycerol-U-$^{14}$C uptake by PA-$1_L$ induced 0.5 hours (▲); one hour (●); two hours (○); three hours (△); four hours (□); and eight hours (⬡).


1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
TIME (minutes)
14C-Glycerol, n moles / mg dry wt of cells

Therefore, a four-hour induction period was adopted for use in further experiments.

TABLE II

INDUCTION PATTERN FOR GLYCEROL DEGRADATIVE ENZYMES OF PA-$1_L$ IN RELATION TO GROWTH

| Time Hours | Specific Activities nmoles/min/mg of protein | | Growth O. D. of 1/10 dilution |
|---|---|---|---|
| | Glycerol Kinase | L-α-glycerophosphate dehydrogenase | |
| 0.5 | 1.06 | 3.14 | 0.11 |
| 1 | 4.95 | 5.56 | 0.12 |
| 2 | 4.48 | 7.94 | 0.23 |
| 3 | 8.02 | 8.73 | 0.35 |
| 4 | 7.86 | 9.72 | 0.38 |
| 8 | 3.05 | 3.19 | 0.42 |

Samples of PA-$1_L$ cells were the same as those described in Figure 4.

In order to check for induction of glycerol uptake activity by other growth substrates, PA-$1_L$ cells were grown in minimal media containing lactate, histidine, succinate, or glucose, respectively, with each carbon source at a concentration of 0.5 per cent. $^{14}$C-Glycerol transport was measured and then part of each culture was shocked.

Shock fluids were assayed for glycerol binding activity by equilibrium dialysis. The remaining whole cells were induced for four hours in 0.5 per cent glycerol minimal medium A, after which a second transport measurement was made.

As seen in Figure 5a, succinate-grown cells took up an amount of glycerol equivalent to only nine per cent of the induced level after a period of 10 minutes. This uptake appeared linear. Also, shock fluid from succinate-grown cells displayed no capacity for glycerol binding. All other levels of glycerol transport by uninduced cells were higher than that found for succinate-grown cells but less than their respective levels after induction (Figure 5b-5d). Shock fluids from histidine-, lactate-, and glucose-grown cells exhibited some glycerol binding activity. These data are indicated on each figure (distribution ratios for equilibrium dialysis). All transport by induced cells appeared approximately the same.

Measurement of glycerol uptake by wild-type cells grown on a number of other substrates was made. Again, each carbon source was added to minimal medium A to a concentration of 0.5 per cent. The results are shown in Figures 6a-6h. It was apparent that none of the substrates fully induced glycerol transport activity. Ethylene glycol-, fructose-, and gluconate-grown cells displayed the lowest levels of uptake. A distribution ratio for glycerol binding by shock fluid from fructose-grown cells was 1.10 indicating a low level of glycerol binding. The remaining substrates tested failed to induce levels of glycerol transport equivalent to or greater than those displayed by glucose- and lactate-grown cells (shown in Figure 5).

Figure 5. Transport of Glycerol-U-$^{14}$C by Uninduced and Induced PA-$1_L$.

Uninduced cells were grown on substrates other than glycerol. A portion of each batch of cells was removed for transport and binding assays, while the rest were washed and induced 4 hr in 0.5 per cent glycerol minimal medium A. A second transport measurement was then made.

The R's with letter subscripts indicate binding ratios for shock fluids from succinate-(S), histidine-(H), lactate-(L), and glucose-grown (G) cells.

Curves for uninduced transport are those with closed symbols. Open symbols represent curves for induced cells.

Succinate-grown (▲); succinate-grown, glycerol-induced (△); histidine-grown (●); histidine-grown, glycerol-induced (o); lactate-grown (⬢); lactate-grown, glycerol-induced (⬡); glucose-grown (■); and glucose-grown, glycerol-induced (□).

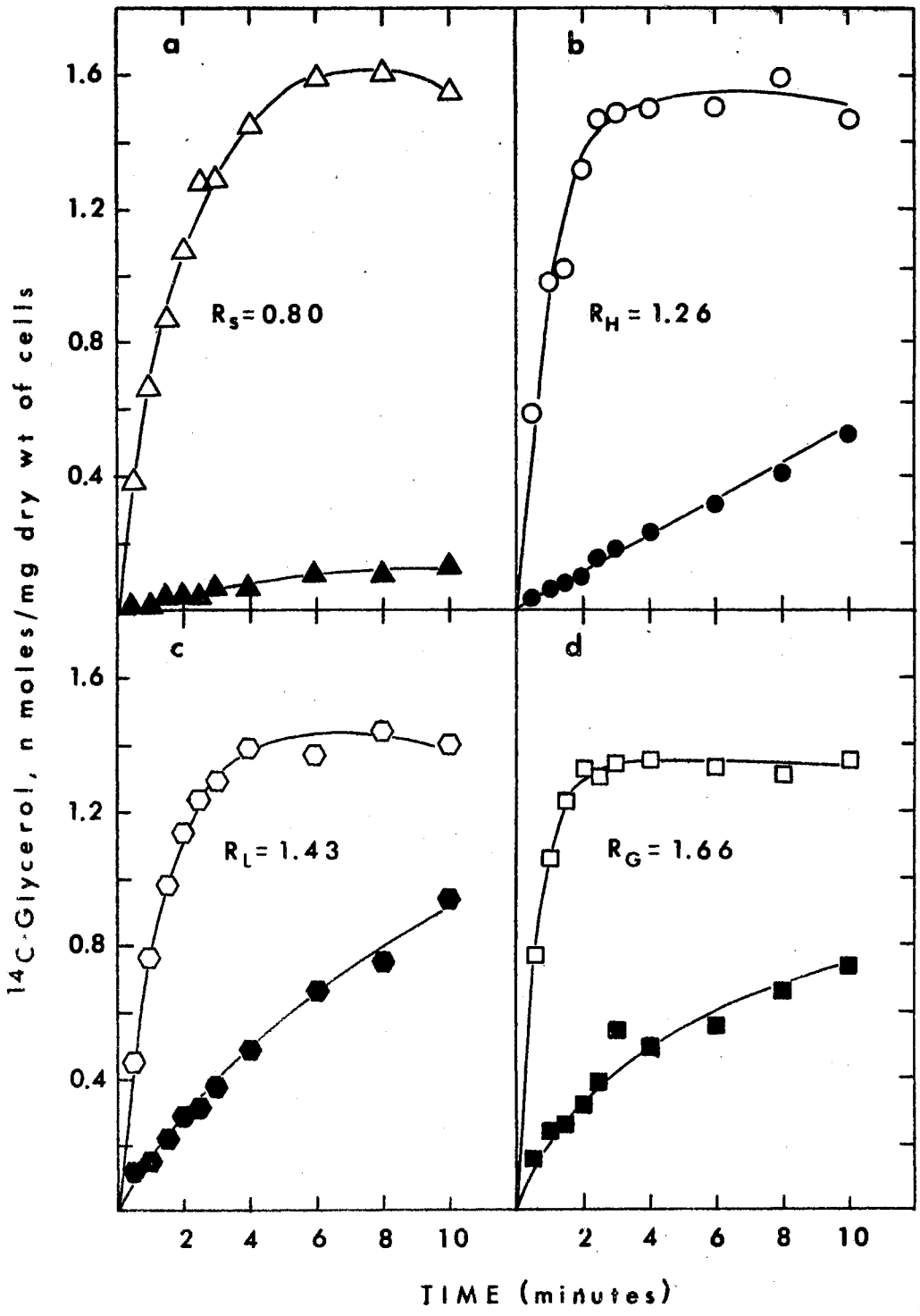
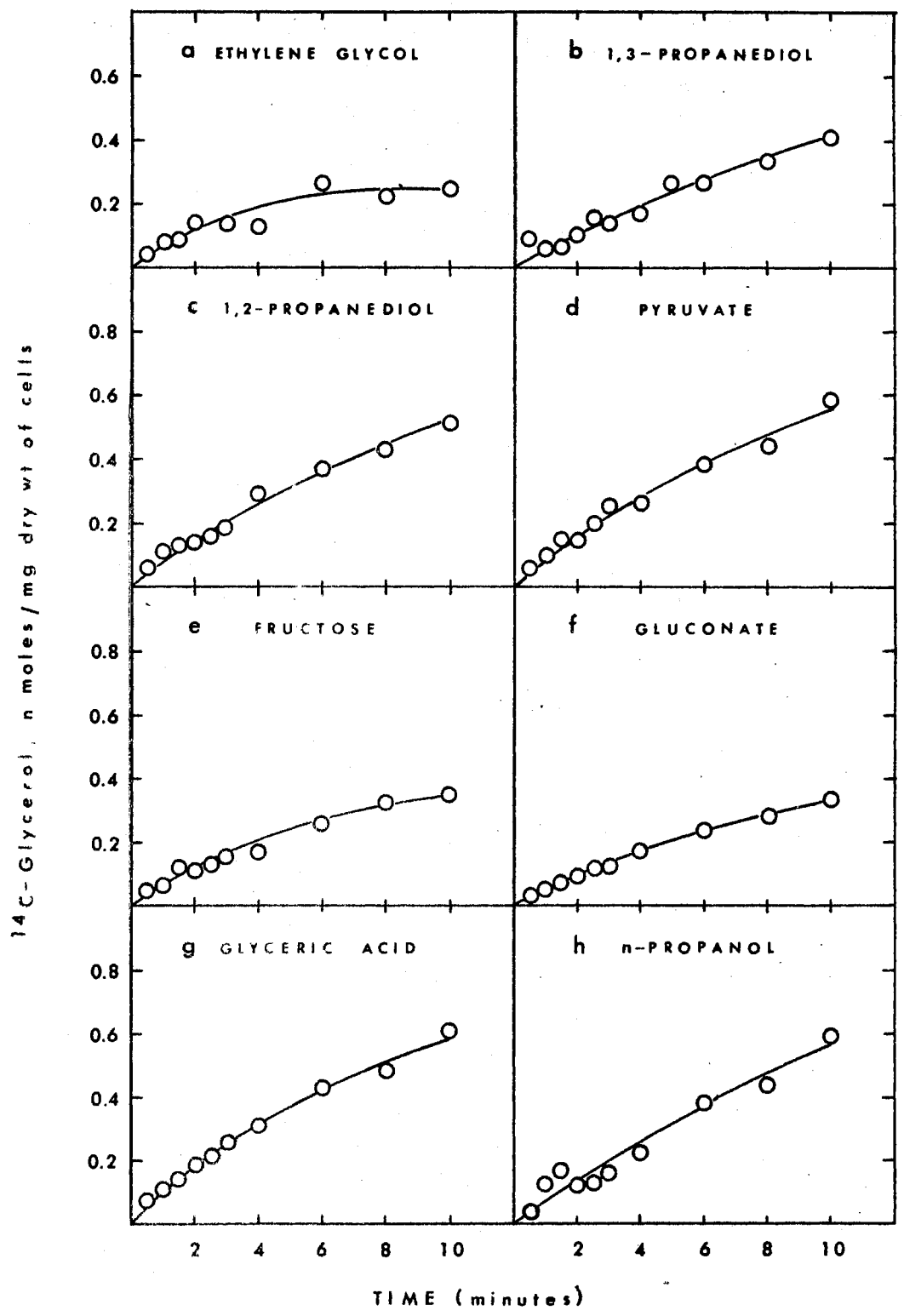

a
b
c
d
$R_S = 0.80$
$R_H = 1.26$
$R_L = 1.43$
$R_G = 1.66$
1.6
1.2
0.8
0.4
2
4
6
8
10
$^{14}$C-Glycerol, n moles/mg dry wt of cells
TIME (minutes)

Figure 6. Transport of Glycerol-U-$^{14}$C by Uninduced PA-$1_L$.

Cells were grown in minimal medium A with the carbon sources indicated in each graph. Carbon source concentration was 0.5 per cent. Cells were prepared for transport assays as described in Materials and Methods.


a ETHYLENE GLYCOL
b 1,3-PROPANEDIOL
c 1,2-PROPANEDIOL
d PYRUVATE
e FRUCTOSE
f GLUCONATE
g GLYCERIC ACID
h n-PROPANOL
0.2
0.4
0.6
0.8
2
4
6
8
10
TIME (minutes)
14C-Glycerol, n moles / mg dry wt of cells

## Distinction Between Glycerol Binding Activity and the Activities of the Glycerol Degradative Enzymes

Glycerol binding activity of shock fluids from induced wild-type cells could be attributed to loss of glycerol kinase and/or L-α-glycerophosphate dehydrogenase from shocked cells rather than to the presence of a binding protein specifically involved in glycerol transport. Since Lin has attributed uptake of glycerol in E. coli to facilitated diffusion, with accumulation and retention of the substrate being dependent upon glycerol kinase activity (1970), it was particularly important to distinguish between binding and kinase activities. For this purpose, 600 ml of PA-$1_L$ cells were grown in 0.5 per cent glycerol minimal medium A to 20 per cent transmittance. An extract was prepared from half of the cells by sonication while the remainder were shocked with 0.5 M sucrose-0.05 M Tris-1.0 mg/ml EDTA. The shock pellet was then sonicated. The shock fluid was assayed for glycerol binding, kinase, and dehydrogenase activities. After measuring initial activities, extracts and shock fluid were frozen and thawed twice. Again, binding and enzyme activities were assayed. Table III shows the results. Data for another preparation of shock fluid, treated similarly, are also shown (last line of table).

The two different preparations of shock fluid from glycerol-grown wild-type cells varied in activities demonstrated. One preparation had no measurable kinase activity, while the other had a total glycerol kinase activity equivalent to 0.6 per cent of the total activity of an extract from an equal number of unshocked cells (67 nmoles/min total activity). Neither preparation exhibited L-α-glycerophosphate dehydrogenase activity.

TABLE III

EFFECT OF SHOCKING AND FREEZE-THAWING ON GLYCEROL BINDING ACTIVITY AND ON THE ACTIVITIES OF THE GLYCEROL DEGRADATIVE ENZYMES

| Treatment | Binding (R) | | Glycerol Kinase | | L-α-Glycerophosphate Dehydrogenase | |
|---|---|---|---|---|---|---|
| | Before F-T | After F-T | Before F-T | After F-T | Before F-T | After F-T |
| Extract of Whole Cells* | - | - | 9.64 | 9.64 | 11.20 | 11.20 |
| Extract of Shock Pellet | - | - | 9.40 | 9.40 | 8.10 | 8.10 |
| Shock Fluid** | 3.99 | 1.17 | 6.18 | 6.18 | 0.00 | 0.00 |
| Shock Fluid | 3.74 | 1.24 | 0.00 | 0.00 | 0.00 | 0.00 |

Abbreviations are as follows: F-T, freeze-thaw.
PA-1$_L$ cells were grown in 0.5 per cent glycerol minimal medium A in all cases.
Enzyme activities are expressed as nmoles/min/mg protein.
Binding is expressed as the distribution ratio (R).
*125 ml total volume, 11,616 nmoles/min total kinase activity.
**20 ml total volume, 67 nmoles/min total kinase activity.

The two activities detected (binding and kinase) exhibited differences in stability to freezing. The shock fluid containing glycerol kinase activity retained this activity after freeze-thaw but lost almost all (94 per cent) of its binding activity. It was noted repeatedly during the course of this investigation that, in cell-free extracts, both glycerol kinase and L-α-glycerophosphate dehydrogenase activities were stable to freeze-thaw.

In order to distinguish enzyme activities from binding activity by other criteria, a comparison was made of effects of numerous compounds on activities of extracts and shock fluids. Results are listed in Table IV. In all cases, the compound being tested and the appropriate amount of extract or shock fluid were mixed and preincubated at least 15 minutes before adding the remaining constituents and assaying. No significant alteration of pH was observed after addition of any of the compounds except in the case of hydrazine.

Sucrose at a concentration of 0.5 M and 1.0 mg/ml EDTA, which were components of the binding assay, were found to have no effect on either glycerol kinase or L-α-glycerophosphate dehydrogenase activity. Known inhibitors of energy production via electron transport or substrate level phosphorylation had varied effects. Sodium azide at 300 mM and sodium arsenate as high as 100 mM showed no substantial inhibition of either enzyme. Yet both, at a 30 mM concentration in shock fluid, eliminated glycerol binding. Sodium cyanide, a constituent of the dehydrogenase assay, also caused complete inhibition of glycerol binding and inhibited the kinase at a 10-fold higher concentration. 2,4-Dinitrophenol, on the other hand, had no significant effect on any of the activities. It may be noted that 2,4-dinitrophenol is a highly

TABLE IV

EFFECT OF POSSIBLY INHIBITORY COMPOUNDS ON GLYCEROL BINDING ACTIVITY AND ON ACTIVITIES OF THE GLYCEROL DEGRADATIVE ENZYMES

| Possible Inhibitor | Conc. of Inhibitor in Enzyme Assay | Enzyme Activities | | | | Conc. of Inhibitor in Binding Assay | Binding Activity (R) | |
|---|---|---|---|---|---|---|---|---|
| | | Kinase | | Dehydrogenase | | | | |
| | | Without | With | Without | With | | Without | With |
| Sucrose | 0.5 M | 6.92 | 6.92 | 5.19 | 5.19 | 0.5 M | In | Assay |
| EDTA | 1 mM or 1 mg/ml | 4.70 | 4.70 | 6.33 | 6.32 | 1 mM or 1 mg/ml | In | Assay |
| Sodium Azide | 300 mM | 5.51 | 5.02 | 6.85 | 7.12 | 30 mM | 1.86 | 0.99 |
| Sodium Cyanide | 300 mM | 6.00 | 0.88 | In | Assay | 30 mM | 3.99 | 1.00 |
| 2,4-Dinitrophenol | 100 mM | 6.63 | 5.26 | 4.54 | 3.87 | 10 mM | 3.99 | 4.04 |
| NEM | 10 mM | 6.92 | 5.22 | 5.19 | 0.86 | 1 mM | 1.86 | 0.99 |
| NEM | 100 mM | 6.92 | 0.00 | 5.19 | 0.00 | 1 mM | 1.86 | 1.07 |
| Hydrazine | 0.3 N | In | Assay | 5.19 | 5.50 | 0.3 N | 1.86 | 1.07 |
| Sodium Arsenate | 10 mM | 10.72 | 12.78 | - | - | 30 mM | 1.42 | 1.00 |
| Sodium Arsenate | 100 mM | 10.72 | 12.78 | - | - | - | - | - |
| Sodium Fluoride | - | - | - | - | - | 30 mM | 1.46 | 1.46 |
| Oligomycin | - | - | - | - | - | $1x10^{-5}$ M | 1.92 | 1.96 |
| Antimycin A | - | - | - | - | - | $1x10^{-5}$ M | 1.56 | 1.55 |
| Mercaptoethanol | 50 mM | 6.92 | 6.04 | - | - | - | - | - |

Abbreviations are as follows: Conc., concentration; NEM, N-ethylmaleimide; EDTA, disodium (Ethylenedinitrilo)tetracetate.
Extracts and shock fluids were prepared from PA-$1_S$ grown in 0.5 per cent glycerol minimal medium A.
Without and with indicate absence and presence of inhibitor.
Enzyme activities are expressed as nmoles/min/mg protein.

colored compound (yellow). When this compound was added to cuvettes for enzyme assay at 340 nm and 550 nm, the slit opened almost completely. Of course, this reduced the sensitivity and, more than likely, accounted for the apparent reduction of kinase and dehydrogenase activities. Therefore, it was assumed that the slight decrease in enzyme activities after addition of 2,4-dinitrophenol could be ignored. Hydrazine, a highly reactive compound and a necessary constituent of the glycerol kinase assay, seemed to stimulate L-α-glycerophosphate dehydrogenase activity but inhibited glycerol binding by approximately 92 per cent.

The sulfhydryl reagent, N-ethylmaleimide (NEM), has been shown to inhibit transport of galactose, leucine (Anraku, 1968c), and β-galactosides (Fox and Kennedy, 1965) in E. coli. Tsay (1971) had reported an inhibitory effect of NEM on glycerol transport by P. aeruginosa but found no significant inhibition of glycerol catabolic enzymes assayed in an extract from glycerol-grown PA-1 whole cells pretreated for 15 minutes with NEM before sonication. In the present study, NEM and cell-free extract were preincubated. At the end of 15 minutes, excess NEM was inactivated by addition of 50 mM mercaptoethanol before other components of the assay mixture were added. It was noted that mercaptoethanol alone inhibited glycerol kinase by approximately 15 per cent. Therefore, the control activity for NEM should be 6.04 nmoles/min/mg rather than 6.92 nmoles/min/mg. Then, NEM at a concentration 10 times greater than the concentration used to inhibit transport or binding activity decreased kinase activity by only 14 per cent.

Oligomycin, antimycin A, and sodium fluoride were not tested in cell-free extracts but did not have a detrimental effect on glycerol binding.

The data cited above seem adequate to differentiate binding from kinase and dehydrogenase activities, especially since binding of the substrate would be essential for catalytic activity. However, it might be argued that cofactors required for kinase activity, which were present in the kinase assay but not in the binding assay, would be capable of reactivating the enzyme so that inhibition would be observed in the binding assay but not in that for enzyme activity. If such were the case and the binding activity of the shock fluid were actually due to the presence of glycerol kinase, the cofactors of the kinase reaction should provide protection against inhibition of binding. The data in Table V show that addition of $MgCl_2$ and ATP, separately or in combination, had no protective effect when azide was added to the equilibrium dialysis cell.

## Isolation of Mutants

To add further support to the hypothesis that glycerol binding and glycerol catabolic enzymes are distinctive entities, glycerol negative mutants were isolated and characterized.

### Enrichment Using D-Cycloserine (DCS)-Carbenicillin (CAR)

Glycerol-grown nitrosoguanidine-treated PA-$1_L$ cells were obtained from Floyd E. White, Jr. After washing with minimal medium B, these cells were suspended to approximately 1 X $10^8$ cells per ml in 0.5 per cent glycerol minimal medium A. Eight milliliters of the suspension

TABLE V

EFFECT OF $MgCl_2$ AND ATP UPON INHIBITION OF BINDING BY SODIUM AZIDE IN SHOCK FLUID

| Additions | Glycerol Binding (R) | Per Cent Inhibition |
|---|---|---|
| None | 1.86 | - |
| Sodium Azide | 0.99 | 100 |
| $MgCl_2$ | 2.01 | - |
| $MgCl_2$, Sodium Azide | 1.00 | 100 |
| ATP | 1.43 | - |
| ATP, Sodium Azide | 1.03 | 93 |
| $MgCl_2$, ATP | 1.99 | - |
| $MgCl_2$, ATP, Sodium Azide | 1.03 | 97 |

Concentrations were: Sodium azide, 30 mM; $MgCl_2$, 60 mM; ATP, 1.67 mM.

Shock fluid was prepared from PA-$1_S$ grown in 0.5 per cent glycerol minimal medium A.

were aseptically transferred to a sterile 20 mm tube and placed on a shaker at 37 C for six hours, allowing time for wild-type cells to begin growth. Then, 0.1 ml of a freshly-prepared solution containing 4 mg DCS and 0.8 mg CAR (final concentrations equalling 500 μg/ml DCS and 100 μg/ml CAR) was added to the tube (Heath, 1971). The culture was returned to the shaker where incubation was continued for 12 hours. The cells were washed and resuspended in eight ml of minimal medium B, after which 0.1-ml aliquots of a 10-fold dilution series were spread onto glucose minimal medium A plates which were incubated at 37 C until colonies were large enough to pick. The remaining cell suspension was centrifuged and the cells were resuspended in eight ml of 0.5 per cent glucose minimal medium A in a sterile 20 mm tube and allowed to grow to log phase (about 0.35 O.D.). This cell suspension containing mutants and wild-type cells was washed twice in minimal medium B and resuspended to 1 X $10^8$ cells per ml in 0.5 per cent glycerol minimal medium A for recycling (second enrichment cycle) through the DCS-CAR enrichment. The isolation procedure with plating after each enrichment cycle was continued through three such cycles.

Selection and Detection of Mutants

All colonies were picked from second and third cycle plates (containing no more than 300 colonies per plate) and spotted on 0.5 per cent glucose minimal medium A master plates. After incubation for approximately 24 hours at 37 C, the glucose plates were replicated, using the replica-plating method of Lederberg and Lederberg (1952), to 0.5 per cent glycerol minimal medium A plates, 0.5 per cent lactate minimal medium A plates, 0.5 per cent glucose minimal medium A plates,

and nutrient agar plates, respectively. Presumptive mutants were recognized by their failure to grow on glycerol plates after at least 48 hours. Growth on the other two types of minimal medium A plates was desirable while growth on the nutrient agar plate was a necessity. For confirmation, a small sample of the presumptive mutant colony was picked from the glucose replica plate and respotted on glycerol, lactate, and glucose minimal media A plates. Stock cultures of the confirmed glycerol negative mutants were prepared by transferring a portion of the glucose-grown colony to a nutrient agar slant.

After growth of stock cultures, the slants were washed with 2 ml of minimal medium A, aliquots of this wash being used to inoculate liquid test cultures. Sterile, 18mm-diameter tubes containing 8 ml of glycerol, glucose, or lactate liquid minimal medium A, respectively, (0.5 per cent carbon sources) were each inoculated with one drop of slant wash from a 0.1 ml pipette, i.e., about 0.03 ml. Glycerol negative mutants were expected to grow to stationary phase in 12 to 18 hours on glucose and lactate liquid media and to display a per cent transmittance greater than 90 after 60 hours incubation on glycerol liquid medium.

Glycerol negative mutant GA-73 was isolated by this procedure and was used in this study along with AH-8 and PA-1-623.

## Characterization of Mutants

### Glycerol Binding and Enzyme Activities

The previously-mentioned mutants were characterized as to the capacity of their shock fluids to bind glucose and glycerol and as to

the specific activities of their glycerol kinase and L-α-glycerophosphate dehydrogenase. While assaying the wild-type variants (PA-$1_L$ and PA-$1_S$) for control levels, it was noted that not only was there no significant difference between the enzyme activities in the two variants, but that there was essentially no difference between enzyme levels in cell-free extracts from sonicated whole cells and from sonicated shock pellets. Therefore, all strains were grown with aeration in large batches (600 ml when induction was required; 300 ml with combination substrates). The 600-ml portions of uninduced cells were divided into two parts, one-half being induced while the other half was shocked. The shock pellet was frozen and then treated as in preparation of cell-free extracts (Materials and Methods). After the remaining cells had been induced they were treated identically. In this way, all data were obtained from aliquots of the same batch of cells. Results are presented in Table VI. For clarification, the numbers in parentheses under kinase indicate the activity after four minutes of reaction. Radioactive assays of commercial glycerol kinase, L-α-glycerophosphate dehydrogenase, and these enzymes in cell-free extracts (following the same protocol as used in spectrophotometric assays and identifying products by chromatography) confirmed the characterization of PA-1, GA-73, and AH-8.

Shock fluid from GA-73 bound both glycerol and glucose. This mutant displayed L-α-glycerophosphate dehydrogenase activity but lacked glycerol kinase. In most cases, the small initial kinase activity dropped to zero. Also, GA-73 displayed dehydrogenase activity when grown in the presence of glucose but not when grown in lactate or succinate.

TABLE VI

BINDING AND ENZYME ACTIVITIES OF GLYCEROL-NEGATIVE MUTANTS

| Strain | Substrate(s)/Inducer | Shock Fluid Binding | | S.F. Prot. Conc. | Enzyme Activities | |
|---|---|---|---|---|---|---|
| | | Glycerol (R) | Glucose (R) | | Kinase | Dehase |
| PA-$1_L$ | Glycerol | 3.34 | 1.98 | 0.47 | 11.75 | 12.95 |
| | Glucose | 1.86 | 2.03 | 0.50 | 0.63 | 0.18 |
| | Lactate | 1.43 | 1.63 | 0.49 | 0.29 | 0.00 |
| | Succinate | 0.87 | 2.03 | 0.45 | 0.34 | 0.00 |
| | Glucose/Glycerol | 3.33 | - | 0.50 | 6.38 | 10.96 |
| | Lactate/Glycerol | 2.11 | - | 0.47 | 3.87 | 7.28 |
| | Succinate/Glycerol | 2.37 | - | 0.52 | 6.29 | 11.07 |
| | Glucose-Glycerol | - | - | - | 6.72 | 11.23 |
| | Lactate-Glycerol | 1.48 | - | 0.48 | 0.74 | 3.55 |
| | Succinate-Glycerol | - | - | - | 8.68 | 7.08 |
| GA-73 | Glucose | 1.64 | 2.73 | 0.41 | 0.20 (0.00) | 3.84 |
| | Lactate | 1.71 | 2.01 | 0.38 | 0.67 (0.00) | 0.81 |
| | Succinate | 1.09 | 2.10 | 0.62 | 0.14 (0.00) | 0.35 |
| | Glucose/Glycerol | 2.20 | 1.97 | 0.44 | 0.65 (0.26) | 3.83 |
| | Lactate/Glycerol | 1.53 | 2.90 | 0.44 | 0.91 (0.00) | 2.78 |
| | Succinate/Glycerol | 1.07 | 2.17 | 0.34 | 1.77 (0.54) | 1.11 |
| | Glucose-Glycerol | 1.43 | 2.00 | 0.48 | 0.22 (0.00) | 6.46 |
| | Lactate-Glycerol | 1.31 | 1.11 | 0.37 | 0.69 | 2.96 |
| | Succinate-Glycerol | 1.12 | 1.98 | 0.32 | 0.00 | 2.15 |
| AH-8 | Glucose | 1.04 | 1.31 | 0.46 | 4.60 | 0.10 |
| | Lactate | 0.99 | 1.36 | 0.38 | 4.20 | 0.00 |
| | Succinate | 0.62 | 1.27 | 0.29 | 7.50 | 0.00 |
| | Glucose/Glycerol | 0.95 | 1.27 | 0.44 | 4.60 | 0.00 |

TABLE VI (Continued)

| Strain | Substrate(s)/Inducer | Shock Fluid Binding | | S.F. Prot. Conc. | Enzyme Activities | |
|---|---|---|---|---|---|---|
| | | Glycerol (R) | Glucose (R) | | Kinase | Dehase |
| AH-8 | Lactate/Glycerol | 1.15 | 2.87 | 0.36 | 4.20 | 0.00 |
| | Succinate/Glycerol | 1.84 | 3.09 | 0.32 | 3.40 | 0.00 |
| | Glucose-Glycerol | 1.23 | 1.38 | 0.35 | 4.40 | 0.00 |
| | Lactate-Glycerol | 0.55 | 1.11 | 0.50 | 2.50 | 0.00 |
| | Succinate-Glycerol | 1.01 | 0.96 | 0.87 | 2.60 | 0.00 |
| PA-1-623 | Glucose | 0.68 | 0.86 | 0.41 | 7.80 | 0.14 |
| | Lactate | 1.15 | 1.11 | 0.31 | 14.22 | 0.00 |
| | Succinate | 0.89 | 1.18 | 0.44 | 4.28 | 0.12 |
| | Glucose/Glycerol | 1.17 | 1.02 | 0.24 | 9.28 | 0.35 |
| | Lactate/Glycerol | 0.91 | 1.31 | 0.26 | 9.98 | 0.26 |
| | Succinate/Glycerol | 1.09 | 1.21 | 0.21 | 2.24 | 0.00 |
| | Glucose-Glycerol | 1.02 | 1.36 | 0.29 | 7.72 | 0.00 |
| | Lactate-Glycerol | 1.06 | 1.13 | 0.30 | 7.22 | 0.00 |
| | Succinate-Glycerol | 1.09 | 1.11 | 0.50 | 7.66 | 0.00 |

Enzyme activities are expressed as nmoles/min/mg protein.
Abbreviations are as follows: S.F. Prot. Conc., Shock fluid protein concentration (mg/ml); Kinase, Glycerol Kinase; Dehase, L-α-glycerophosphate dehydrogenase; Glucose/Glycerol, glucose-grown glycerol-induced; Glucose-Glycerol, grown in the presence of both glucose and glycerol.

AH-8 was characterized as having glycerol kinase activity and partial binding activity, but lacking L-α-glycerophosphate dehydrogenase activity. In contrast to the wild-type, this strain had glycerol kinase activity even when grown in the presence of glucose, lactate, or succinate.

PA-1-623, on the other hand, lacked glycerol binding activity but had low levels of glucose binding activity. It had elevated glycerol kinase activity and lacked L-α-glycerophosphate dehydrogenase activity. Again, kinase activity was apparent when the cells were grown in the presence of glucose, lactate, or succinate.

Glycerol-U-$^{14}$C Transport

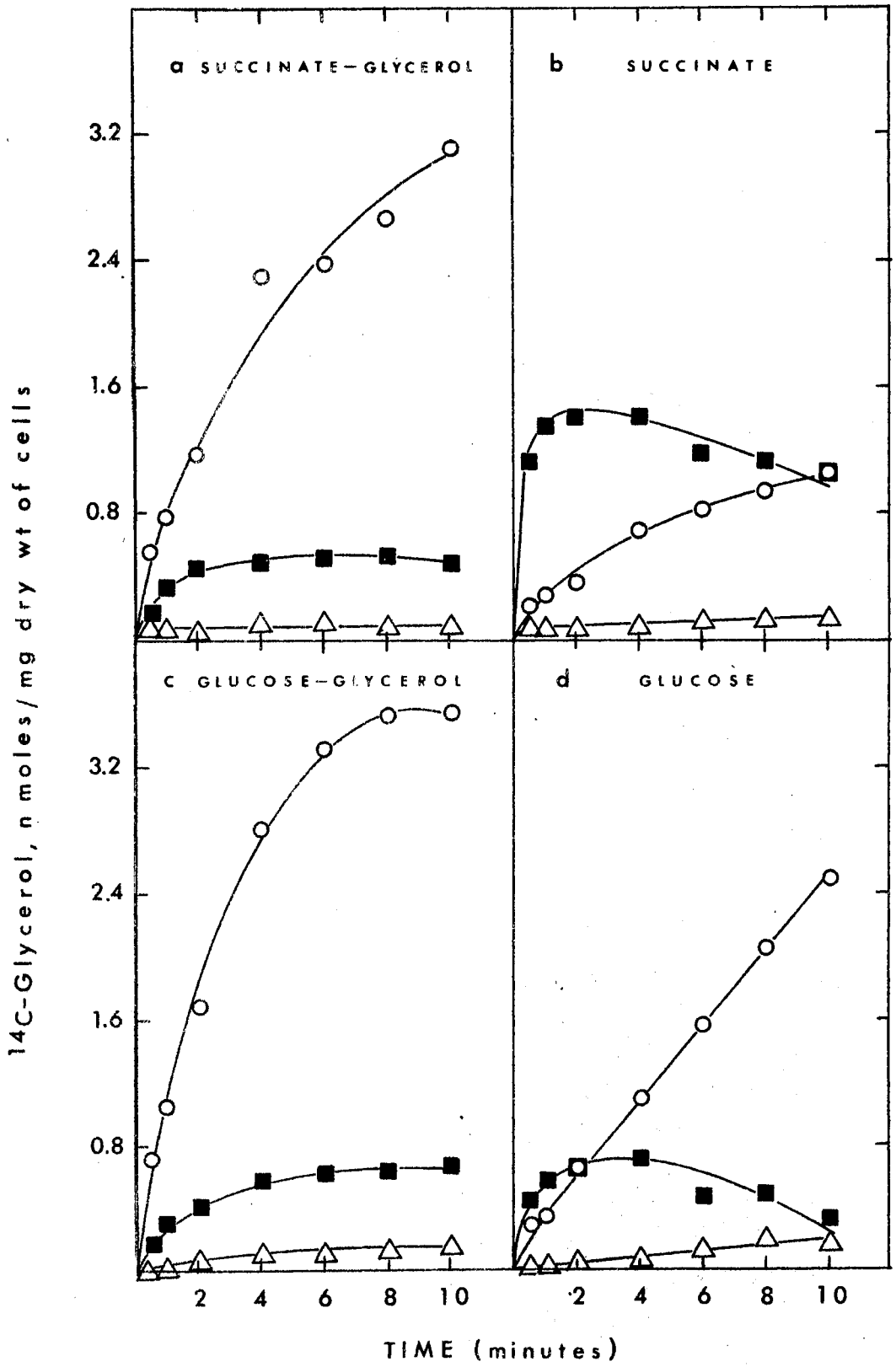
From the previously-mentioned large batches of cells (enzyme and binding data given in Table VI), a 25-ml sample of each mutant cell suspension was tested for its ability to transport glycerol-U-$^{14}$C. All substrate combinations were tested, with each mutant exhibiting similar differences between induced and uninduced batches of cells. Therefore, four representative substrates were chosen for demonstration of transport in the three mutants. Uninduced transport levels were assayed in cells grown in succinate or in glucose. Since it was difficult to induce transport in the glycerol negative strains by the conventional method (aeration for four hours in glycerol minimal medium A), combination substrates were used. Combinations of 0.5 per cent glucose plus 0.5 per cent glycerol or 0.5 per cent succinate plus 0.5 per cent glycerol thus served as inducer substrates. Figures 7a-7d show the uninduced and induced transport curves for each of the mutant strains.

Figure 7. Transport of Glycerol-U-$^{14}$C by Mutants AH-8, GA-73, and PA-1-623.

Uninduced mutants were grown on either glucose or succinate, harvested, and assayed for glycerol transport according to the procedures described in Materials and Methods. Induced cells were grown in a glucose-glycerol combination or a succinate-glycerol combination, after which transport ability was measured.

The carbon source used for growth is shown on the individual plots.

Glycerol-U-$^{14}$C transport by AH-8 (■); by GA-73 (o); and by PA-1-623 (Δ).


a SUCCINATE–GLYCEROL
b SUCCINATE
c GLUCOSE–GLYCEROL
d GLUCOSE
3.2
2.4
1.6
0.8
2
4
6
8
10
14C-Glycerol, n moles / mg dry wt of cells
TIME (minutes)

Mutant GA-73, which showed full binding activity (Table VI) displayed a high level of glycerol transport when induced. The level was also significant when this strain was grown in the presence of glucose, but considerably lower when grown in succinate. This difference correlated with that in enzyme activities.

On the other hand, PA-1-623 appeared to take up little or no glycerol. This was in agreement with data showing that it had no detectible glycerol binding capacity (Table VI).

Glycerol transport by AH-8 was unusual. When induced, it displayed partial transport ability as expected from its binding activities. It was similar to the wild-type in that a plateau was attained. On the other hand, uninduced cells appeared to have increased initial transport followed by a loss of $^{14}C$ to below the 30-second value at the end of 10 minutes. No level plateau was observed. When grown on single substrates or induced for four hours after growth on single substrates, the later in the growth cycle that AH-8 cells were harvested, the greater was the initial uptake value. However, the plateau height (difference between the initial 30-second value and the highest point in the plateau) remained constant for all AH-8 glycerol transport curves as demonstrated in Figure 7.

Although PA-1-623 and AH-8 seemed to be similar enzymatically, their binding and transport capacities differed significantly. This suggested a direct correlation between binding and transport and provided further evidence negating the hypothesis that glycerol transport was dependent upon glycerol kinase activity.

Previously, data shown in Figure 3b had indicated that osmotic shocking of wild-type cells removed binder and simultaneously decreased

transport of glycerol when fragile rods were protected in 0.5 M sucrose. This procedure was repeated with AH-8 and PA-1-623, with results shown in Figure 8. Osmotic shocking of AH-8 reduced glycerol transport by approximately 52 per cent whereas the small amount of transport by PA-1-623 was unaffected.

In addition, Figure 8 shows transport by the two mutant strains resuspended in minimal medium B, where they were unprotected. The optical density had been adjusted to 0.35 before measurement of transport. Afterward, the O.D. had decreased to between 0.22 and 0.26 indicating that some lysis had occurred. It was noted that, in both instances, as lysis occurred, glycerol uptake increased until it far surpassed that displayed by whole cells. This suggested that cells lysed prior to measurement of transport could produce a pseudo-uptake curve which might show a high initial rate (value) and might, or might not, appear normal in the later phases. It also indicated that PA-1-623 could take up glycerol if the substrate could gain access to the cell, i.e., when the permeability barrier was altered by osmotic shocking in the absence of an osmotically protective medium.

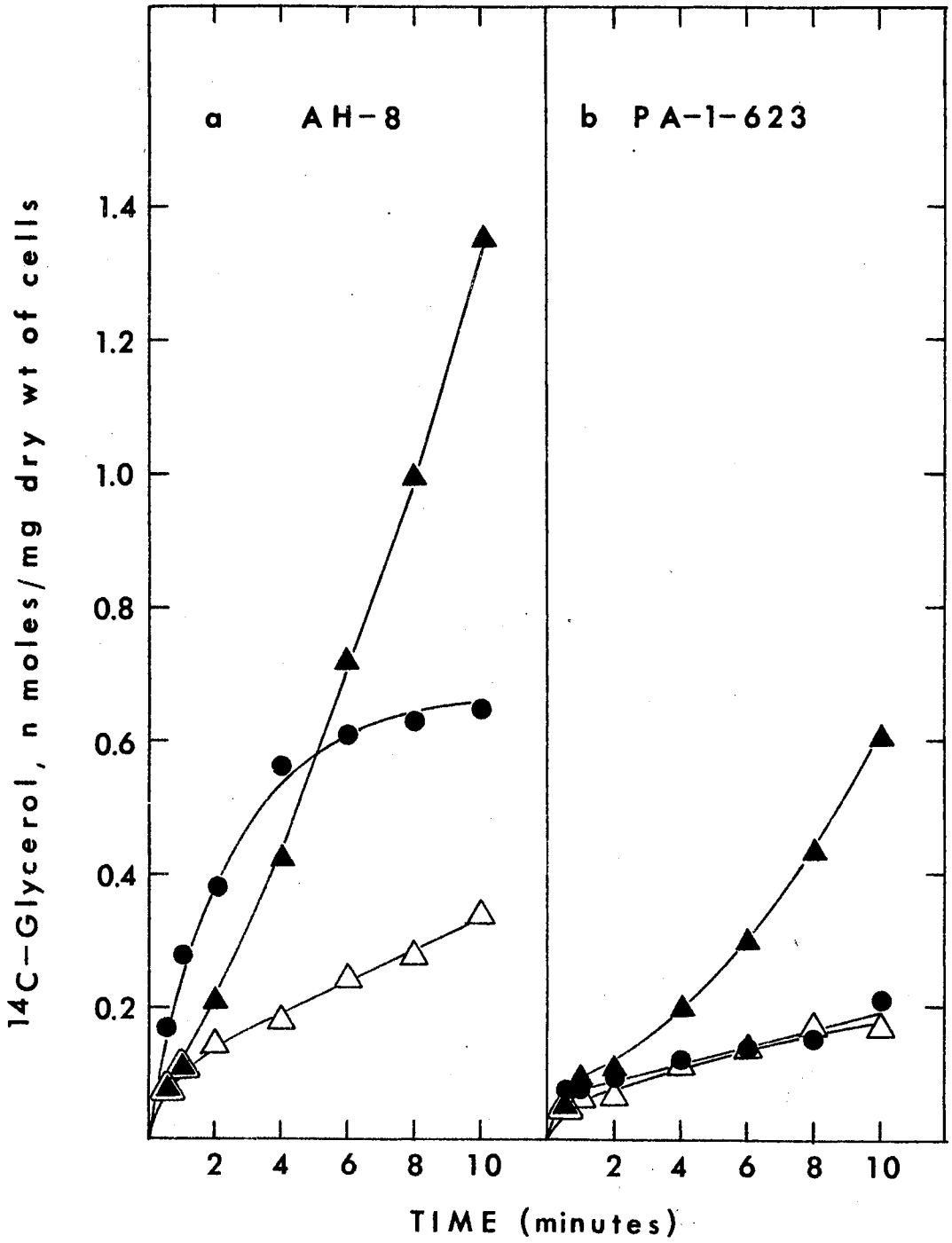
pH Profiles for Mutants as Compared With Wild-Type

The final aspect of mutant characterization involved partial purification of binding proteins and determination of pH profiles for binding of glycerol and glucose. Mutant cells were grown on the glucose plus glycerol combination substrate. Controls were run using wild-type cells grown in the presence of several different substrates and it was found that, in this respect, there was a difference between the large and small colony variants. First, the partially purified

Figure 8. Transport of Glycerol-U-$^{14}$C by AH-8 and PA-1-623 as Whole Cells, Protected Fragile Rods, and Unprotected Fragile Rods.

AH-8 and PA-1-623 were grown in the combination substrate, glucose-glycerol. A portion of each suspension was tested for ability to transport glycerol. The remaining cell suspension, in each case, was centrifuged, washed, and shocked according to previously-described procedures. Part of each shock pellet was resuspended to 45 per cent transmittance in 0.5 M sucrose (protected fragile rods) and assayed for $^{14}$C-glycerol transport. A second portion of each shock pellet was resuspended to 45 per cent transmittance in minimal medium B (unprotected fragile rods) and treated as whole cells during a transport measurement, i.e. filters were washed with minimal medium B.

a. and b. Glycerol-$^{14}$C transport by whole cells (●); protected fragile rods (△); and unprotected fragile rods (▲).


a AH-8
b PA-1-623
14C-Glycerol, n moles/mg dry wt of cells
1.4
1.2
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
TIME (minutes)

protein precipitate obtained from PA-$1_S$ was white and flaky, dissolving easily in phosphate buffer, whereas that isolated from PA-$1_L$ appeared yellowish and translucent and was quite difficult to dissolve. Secondly, when the partially purified binding protein solutions (PPBPS) were assayed for a rough estimate of lipopolysaccharide (LPS), it was noted that the same volume of PA-$1_L$ PPBPS contained three times more LPS than that from PA-$1_S$ (Judy A. Smith, personal communication). AH-8 displayed a high value, similar to that of PA-$1_L$ (approximately 136 µg/ml whereas the LPS values for GA-73 and PA-1-623 were lower (80 and 48 µg/ml respectively).

Since each peak in Figures 9-11 occurs as a result of increased binding activity at specific pH's and under varying conditions, the term "peak" will be used loosely to denote points of increased glycerol and/or glucose binding, which (for a single substrate) probably represent distinct binding sites that may or may not be located on separate protein molecules.

Figures 9 and 10 represent the pH profiles for control cells and must be considered first. The profile for PA-$1_S$ grown in glycerol minimal medium A (Figure 9b) suggested the presence of two peaks each representing glucose and glycerol binding: one set with a binding optimum at pH 5 and the other at 7. However, upon analysis of Figures 9 and 10, four definite peaks were noted. A possible fifth one must also be considered.

Peak # 1 bound glycerol optimally at pH 7 and was only found at significant levels in the profiles of wild-type cells exposed to glycerol. It appeared that whenever large colony variant cells were used (Figure 9a) or were present to some extent (Figure 10a) peaks

Figure 9. pH Binding Profiles for Partially Purified Binding Proteins of PA-$1_L$ and PA-$1_S$.

Partially purified binding protein was obtained and assayed following the procedure described in Materials and Methods.

Glucose-glycerol-grown cells are cells grown in minimal medium A containing both 0.5 per cent glucose and 0.5 per cent glycerol.

Peaks are identified by numbers.

a. Profile for glucose-glycerol-grown PA-$1_L$.

b. Profile for glycerol-grown PA-$1_S$.

Solid circles (●) denote glycerol binding. Open triangles (△) denote glucose binding.

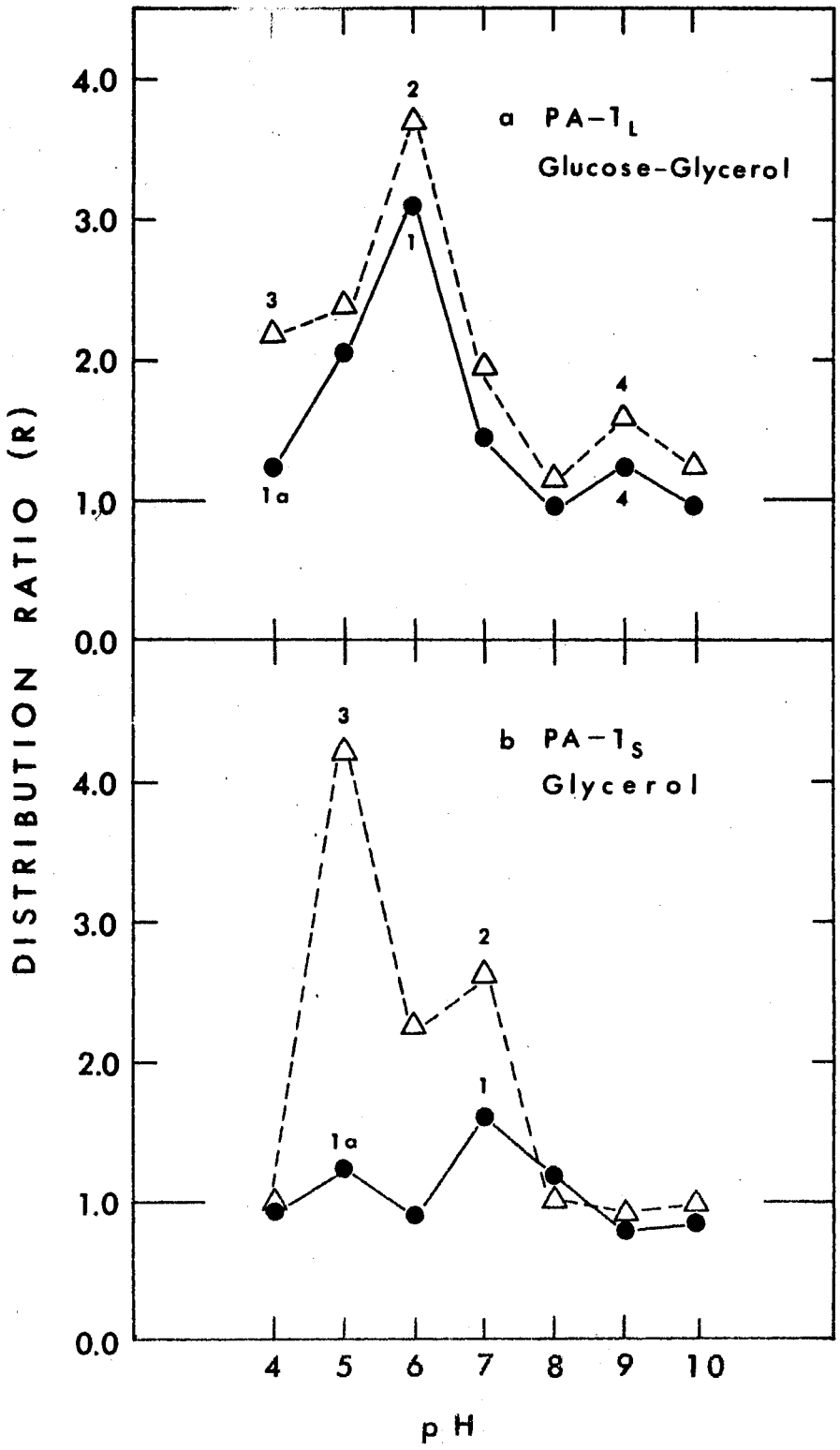

a PA-1L
Glucose-Glycerol
b PA-1S
Glycerol
DISTRIBUTION RATIO (R)
pH
4.0
3.0
2.0
1.0
0.0
4
5
6
7
8
9
10
1
1a
2
3
4

Figure 10. pH Binding Profiles for Partially Purified Binding Proteins of Uninduced Cells.

The experiment was performed as described for Figure 10 except that carbon sources differed.

Glucose-grown PA-$1_S$ was consistently found to be contaminated with PA-$1_L$ (determined from platings). These are designated PA-$1_{S+L}$.

a. Profile for glucose-grown PA-$1_{S+L}$.

b. Profile for succinate-grown PA-$1_S$.

Solid circles (●) denote glycerol binding. Open triangles (△) denote glucose binding.

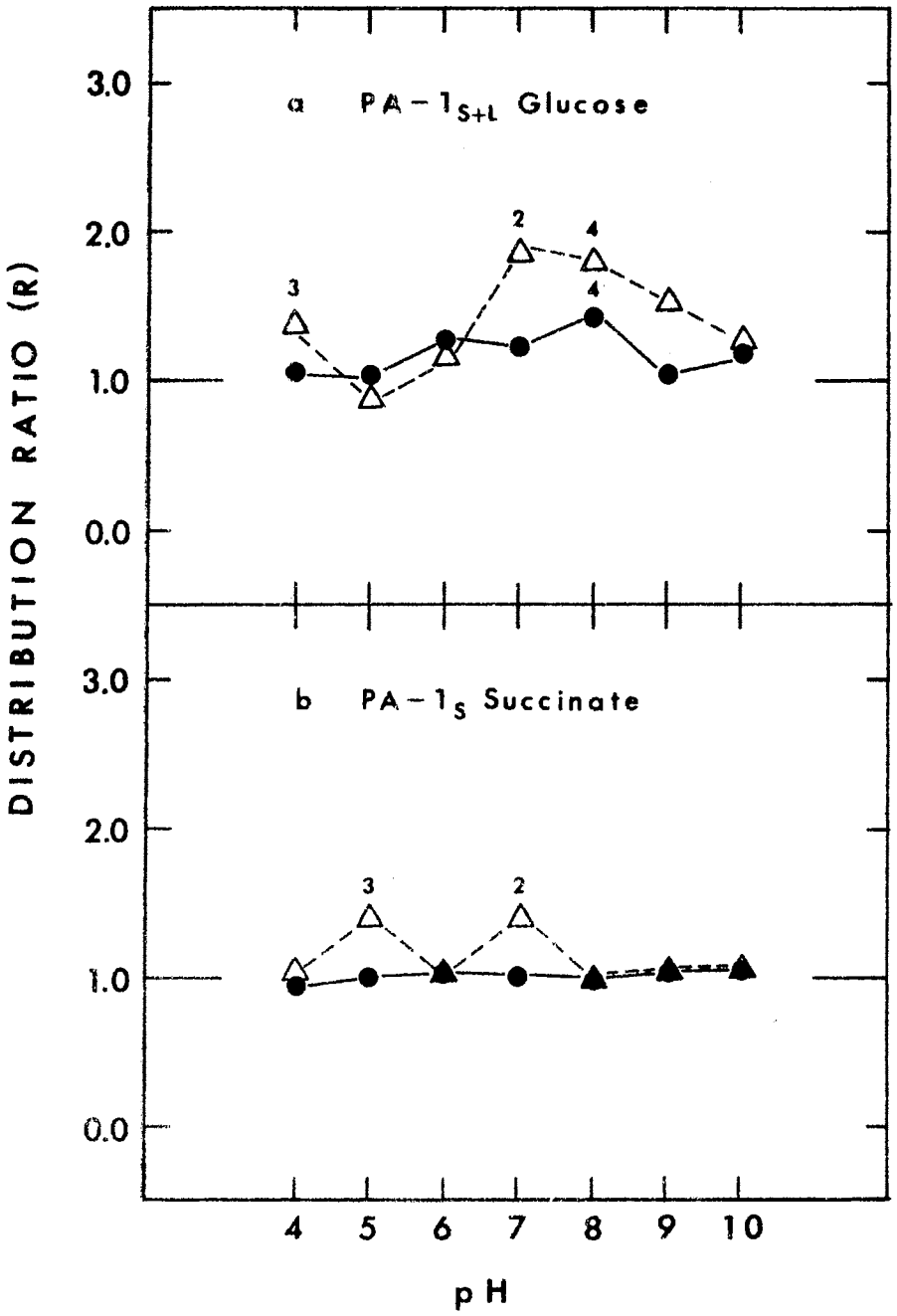

a PA – 1S+L Glucose
b PA – 1S Succinate
DISTRIBUTION RATIO (R)
3.0
2.0
1.0
0.0
4 5 6 7 8 9 10
pH

Figure 11. pH Binding Profiles for Partially Purified Binding Proteins of Mutants.

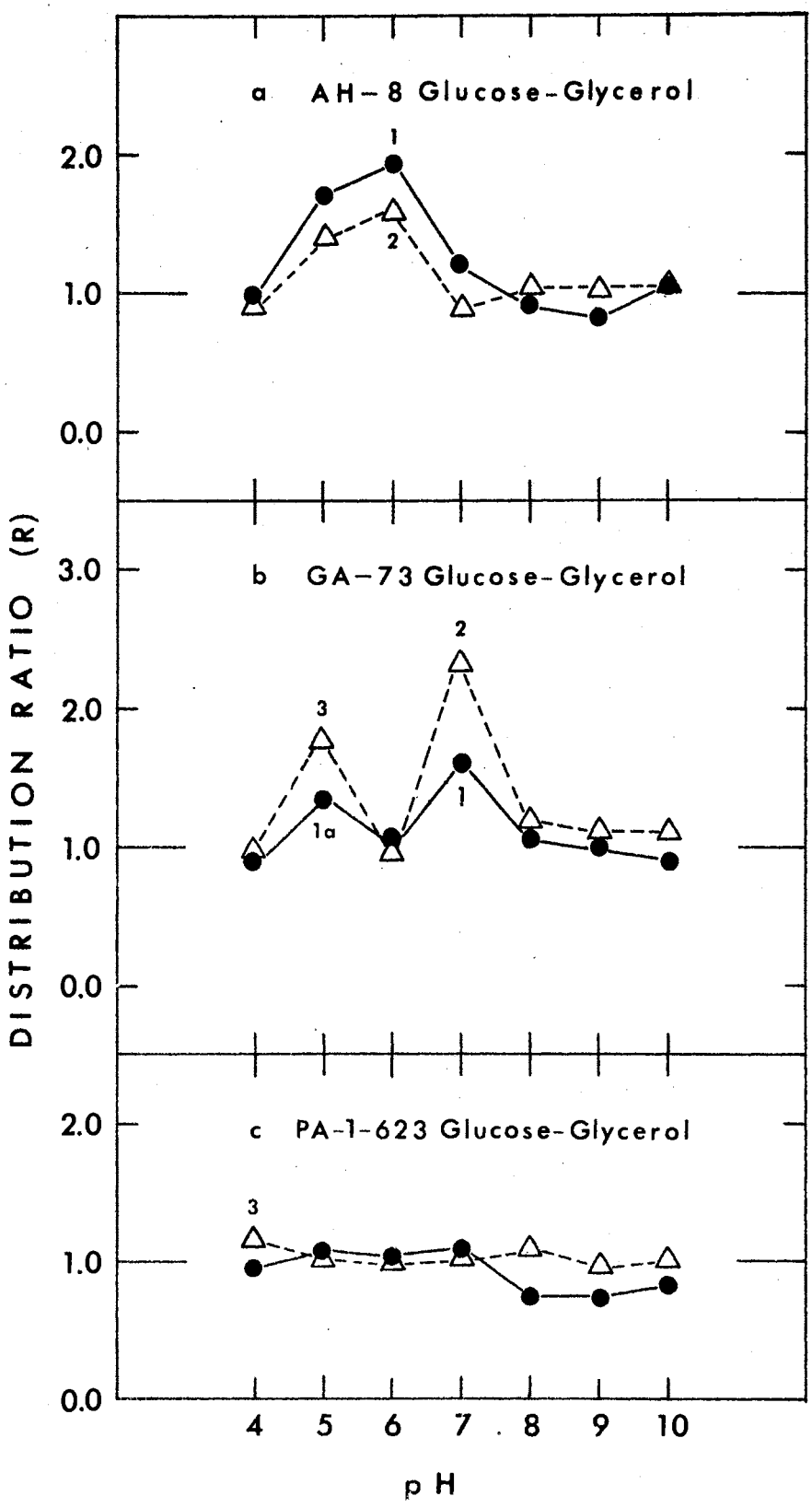
The experiment was performed as described for Figure 10. All mutants were grown in the combination, glucose-glycerol minimal medium A.

a. Profile for AH-8.

b. Profile for GA-73.

c. Profile for PA-1-623.

Solid circles (●) denote glycerol binding. Open triangles (△) denote glucose binding.


a AH-8 Glucose-Glycerol
b GA-73 Glucose-Glycerol
c PA-1-623 Glucose-Glycerol
DISTRIBUTION RATIO (R)
2.0
1.0
0.0
3.0
2.0
1.0
0.0
2.0
1.0
0.0
1
2
3
1a
1
2
3
4 5 6 7 8 9 10
pH

indicating pH optima were shifted; the apparent direction of shift was downward, i.e., from pH 7 to pH 6 for peak # 1. Peak # 1a with an optimum for glycerol binding at pH 5 in PA-$1_S$ PPBPS and pH 4 in PA-$1_L$ PPBPS was also missing when glycerol was not present.

Peak # 2, representing a glucose binder, had a pH optimum at pH 7 (shifted to pH 6 in PA-$1_L$ PPBPS). This peak appeared to be present under all growth conditions, even in PPBPS from cells grown in the presence of succinate, although in a small amount in this case (Figure 10b). The PPBPS from a mixed culture (Figure 10a) exhibited some peak # 2 with an optimum at pH 7. It was possible that this sample actually contained more PA-$1_S$ than PA-$1_L$. A second glucose binder with a pH optimum at 5, shifted to pH 4 in PA-$1_L$, was labeled peak # 3. This binder was also apparent under all growth conditions.

It appeared that peak # 4 was possibly a combination glycerol and glucose binder induced by glucose. It did not seem to be present in PPBPS from cells grown in the presence of glycerol or succinate.

Mutant pH binding profiles are shown in Figure 11. All strains were grown in the combination glucose-glycerol substrate. AH-8 appeared to display a pH shift similar to PA-$1_L$. Making that assumption, this mutant could only synthesize two binders, those represented by peaks # 1 and # 2. Nevertheless, this would allow it to bind both glucose and glycerol. GA-73, on the other hand, exhibited a binding profile quite similar to that of PA-$1_S$. This mutant displayed peaks # 1, # 1a, # 2, and # 3, allowing it several means of binding glycerol and glucose. PA-1-623, enzymatically equivalent to AH-8 but transport negative, was shown to lack all binders for both glucose and glycerol. There was a possible low level of peak # 3 and, perhaps, a trace of

# 1 and # 1a, but, for all practical purposes, PA-1-623 was binder negative.

## Determination of Competition

Basically, competition experiments serve to discern whether two or more substrates are bound and transported by the same carrier. Two types of experiment fall into this category. The first involves obtaining shock fluid from cells induced for a specific binder, and then determining whether similar nonradioactive compounds or possible analogues interfere with (reduce) binding of the labeled substrate. The second is useful for checking the binding results. It involves observance of the effect of the previously-mentioned nonradioactive compounds on the transport of the radioactive substrate. In both instances, determination of specificity is the result.

### Binding Competition

Tables VII and VIII present results from binding competition experiments run on PA-$1_{S+L}$. Table VIII contains the same data as Table VII but values are expressed in terms of per cent inhibition of binding by the added compound. For clarification, two controls were run in part of this series of experiments. Untreated shock fluid was removed immediately and tested for binding activity. Then, at the completion of the experiment (approximately 1.5 hr later) a second untreated sample was removed and tested for binding activity. Two ratios were obtained and designated $Control_1$ (immediately removed sample) and $Control_2$ (sample removed at end of experiment). It was apparent that there was a loss of binding activity with time, a factor

TABLE VII

EFFECT OF VARIOUS NONRADIOACTIVE COMPOUNDS ON BINDING OF $^{14}$C-GLYCEROL AND $^{14}$C-GLUCOSE BY SHOCK FLUIDS FROM PA-$1_{S+L}$

| Inhibitor or Competitor | Concentration | Binding Ratio: Glycerol-Grown Binding Glycerol (R) | Binding Ratio: Glycerol-Grown Binding Glucose (R) | Binding Ratio: Glucose-Grown Binding Glycerol (R) | Binding Ratio: Glucose-Grown Binding Glucose (R) |
|---|---|---|---|---|---|
| N-Ethylmaleimide | 1 mM | 0.98 | 1.00 | 1.01 | 0.99 |
| Sodium Azide | 30 mM | 0.99 | 0.97 | 0.93 | 1.06 |
| 2,4-Dinitrophenol | 10 mM | 2.63 | 1.22 | 0.95 | 1.04 |
| Sodium Cyanide | 30 mM | 1.00 | 1.04 | 0.99 | 1.01 |
| Sodium Lactate | 10 mM | 2.21 | 1.37 | 1.01 | 1.36 |
| Sodium Pyruvate | 10 mM | 1.72 | 1.09 | - | 1.77 |
| Glucose | 10 mM | 1.41 | 0.98 | 1.01 | 1.01 |
| Disodium Succinate | 10 mM | 1.63 | 0.98 | 1.13 | 0.77 |
| Fructose | 10 mM | 1.63 | 0.86 | 0.92 | 1.12 |
| Potassium Gluconate | 10 mM | 1.82 | 1.05 | 1.40 | 1.42 |
| Glycerol | 10 mM | 1.03 | 0.87 | 0.98 | 1.14 |
| 1,2-Propanediol | 10 mM | 1.91 | 1.15 | 1.48 | 1.35 |
| 1,3-Propanediol | 10 mM | 2.15 | 1.13 | 0.96 | 1.21 |
| n-Propanol | 10 mM | 1.24 | 1.54 | 2.42 | 1.58 |
| Mannitol | 10 mM | 1.94 | 0.82 | 1.48 | 1.30 |
| 1,2,4-Butanetriol | 10 mM | 2.36 | 1.02 | 1.28 | 1.01 |
| Histidine (Hydrochloride) | 10 mM | 1.58 | 1.10 | 2.40 | 1.22 |
| Ethanol (Absolute) | 10 mM | 2.14 | 1.20 | 2.20 | 1.42 |
| Dulcitol | 10 mM | 1.39 | 1.62 | 0.98 | 1.30 |
| Adonitol | 10 mM | 1.45 | 1.46 | 1.31 | 1.50 |
| Dihydroxyacetone | 10 mM | 1.68 | 1.63 | 1.01 | 1.33 |
| Erythritol | 10 mM | 2.34 | 1.08 | 1.70 | 1.65 |
| Glyceric Acid (Calcium Salt) | 10 mM | 2.46 | 1.39 | 2.20 | 0.86 |
| Sodium Citrate | 10 mM | 2.23 | 1.71 | 1.67 | 1.38 |
| Control$_1$ (Start of Experiment) | No Additions | 2.62 | - | 2.40 | - |
| Control$_2$ (End of Experiment) | No Additions | 1.67 | 1.61 | 1.83 | 1.57 |

TABLE VIII

EFFECT OF VARIOUS NONRADIOACTIVE COMPOUNDS ON BINDING OF $^{14}$C-GLYCEROL AND $^{14}$C-GLUCOSE BY SHOCK FLUIDS FROM PA-$1_{S+L}$

| Inhibitor or Competitor | Concentration | Per Cent Inhibition of Binding | | | |
|---|---|---|---|---|---|
| | | Glycerol-Grown | | Glucose-Grown | |
| | | Binding Glycerol | Binding Glucose | Binding Glycerol | Binding Glucose |
| N-Ethylmaleimide | 1 mM | 100 | 100 | 99 | 100 |
| Sodium Azide | 30 mM | 100 | 100 | 100 | 90 |
| 2,4-Dinitrophenol | 10 mM | 0 | 59 | 100 | 93 |
| Sodium Cyanide | 30 mM | 100 | 93 | 100 | 98 |
| Sodium Lactate | 10 mM | 0 | 49 | 99 | 100 |
| Sodium Pyruvate | 10 mM | 0 | 85 | - | 0 |
| Glucose | 10 mM | 39 | 100 | 99 | 98 |
| Disodium Succinate | 10 mM | 6 | 100 | 84 | 100 |
| Fructose | 10 mM | 6 | 100 | 100 | 79 |
| Potassium Gluconate | 10 mM | 0 | 92 | 52 | 26 |
| Glycerol | 10 mM | 96 | 100 | 100 | 75 |
| 1,2-Propanediol | 10 mM | 0 | 75 | 42 | 39 |
| 1,3-Propanediol | 10 mM | 0 | 79 | 100 | 63 |
| n-Propanol | 10 mM | 64 | 12 | 0 | 0 |
| Mannitol | 10 mM | 0 | 100 | 42 | 47 |
| 1,2,4-Butanetriol | 10 mM | 0 | 97 | 66 | 98 |
| Histidine (Hydrochloride) | 10 mM | 13 | 84 | 0 | 61 |
| Ethanol (Absolute) | 10 mM | 0 | 67 | 0 | 26 |
| Dulcitol | 10 mM | 42 | 0 | 100 | 48 |
| Adonitol | 10 mM | 33 | 25 | 63 | 12 |
| Dihydroxyacetone | 10 mM | 0 | 0 | 99 | 42 |
| Erythritol | 10 mM | 0 | 87 | 16 | 0 |
| Glyceric Acid (Calcium Salt) | 10 mM | 0 | 36 | 0 | 100 |
| Sodium Citrate | 10 mM | 0 | 0 | 19 | 33 |

which had to be considered when analyzing the data. Since $Control_1$ was run on only two sets of binding assays, the per cent inhibition calculations were based on $Control_2$. Therefore, these percentages may actually be low. They are, however, all relatively comparable.

Data shown in Table VIII indicated that binding of glycerol by shock fluid from glycerol-grown cells was inhibited by only a few compounds. Of the three common energy inhibitors and one protein inactivator listed in the upper segment, all except 2,4-dinitrophenol inhibited this binding activity 100 per cent. Addition of nonradioactive glycerol showed the expected results. The only other significant inhibitors of glycerol binding activity were glucose, n-propanol, dulcitol, adonitol, and possibly histidine.

By looking at Table VII one finds that in the presence of several of the compounds which exhibited zero inhibition of glycerol binding according to Table VIII, R values were actually greater than that for $Control_2$. However, these are in the range of values between $Control_1$ and $Control_2$, suggesting that compounds such as sodium lactate, potassium gluconate, 1,2- and 1,3-propanediols, mannitol, 1,2,4-butanetriol, absolute ethanol, erythritol, glyceric acid, and sodium citrate protected the binder from loss of activity during the experiment.

Binding of glucose by shock fluid from glycerol-grown cells was inhibited to some extent by all compounds except dulcitol, dihydroxyacetone, and sodium citrate. Even 2,4-dinitrophenol inhibited this binding activity by more than 50 per cent. Again, as a control, nonradioactive glucose gave the expected complete inhibition.

Shock fluid from glucose-grown $PA\text{-}1_{S+L}$ was also tested for its ability to bind glycerol and glucose in the presence of the same

nonradioactive compounds. The majority of substances checked did cause inhibition and, in this instance, there appeared to be a great deal of correlation between the two sets of data.

In order to obtain a closer analysis of glycerol binding, competition experiments were run on PPBPS adjusted to the optimum pH values for specific peaks. Glycerol-grown PA-$1_S$ cells were shocked, binding proteins were precipitated with ammonium sulfate, and PPBP was isolated by centrifugation. One-half of this PPBP was suspended in 0.05 M phosphate buffer at pH 5 while the remaining portion was suspended in 0.05 M phosphate buffer at pH 7. Then, a small-scale binding competition experiment was run on each PPBPS, testing 10 of the previously-used compounds and dialyzing against $^{14}$C-glycerol. As before, the untreated control samples were taken at the end of the experiment. The results are presented in Table IX.

The binding of glycerol at pH 5 (peak # 1a in Figures 9-11) was completely inhibited by all compounds tested except gluconate, suggesting nonspecificity of this binder. On the other hand, binding at pH 7 (assumed to be peak # 1) was totally inhibited only by glycerol, as expected, and by sodium azide, and sodium cyanide. The remaining compounds produced only partial inhibition, if any.

Competition in Transport of Glycerol-U-$^{14}$C

Most of the compounds listed in Tables VII and VIII were also checked for their effect on $^{14}$C-glycerol transport by wild-type cells. Possible competitors were added to the cell suspension one minute before zero time after which normal transport procedures were followed. In all cases where a stimulative effect was expected, the untreated

TABLE IX

EFFECT OF VARIOUS NONRADIOACTIVE COMPOUNDS ON $^{14}C$-GLYCEROL BINDING BY PPBPS, ADJUSTED TO pH 5 AND pH 7, FROM GLYCEROL-GROWN PA-$1_S$

| Competitor or Inhibitor | Glycerol Binding | | | |
|---|---|---|---|---|
| | R at pH 5 | Per Cent Inhibition | R at pH 7 | Per Cent Inhibition |
| None | 1.47 | - | 1.59 | - |
| Sodium Pyruvate | 0.98 | 100 | 1.91 | 0 |
| Sodium Lactate | 0.95 | 100 | 1.45 | 24 |
| Disodium Succinate | 1.02 | 96 | 1.20 | 66 |
| Fructose | 0.93 | 100 | 1.41 | 30 |
| Glucose | 0.89 | 100 | 1.29 | 51 |
| Potassium Gluconate | 1.90 | 0 | 1.52 | 12 |
| Glycerol | 0.98 | 100 | 1.03 | 95 |
| Sodium Azide | 0.95 | 100 | 1.02 | 97 |
| Sodium Cyanide | 0.93 | 100 | 1.01 | 98 |
| 2,4-Dinitrophenol | 1.02 | 96 | 1.43 | 27 |

Concentrations of competitors or inhibitors were the same as those listed in Tables VII and VIII.

PPBPS is partially purified binding protein solution.

control was run first in the series, and, when an inhibitory effect was expected, the untreated control was run last in the series. This procedure yielded the most conservative estimate of the effect of the added compound. The possible competitors were initially tested on lactate-grown, glycerol-induced PA-$1_L$. After lactate was found to induce at least some glycerol binder, several of the compounds (lactate, glucose, n-propanol, gluconate, succinate, glycerol, and dihydroxyacetone) were rechecked with glycerol-grown PA-$1_S$. The results were essentially the same and supported the previous observation that only a few substances produced significant effects on glycerol transport. Representative curves for those that did have an effect are shown in Figures 12a-12d.

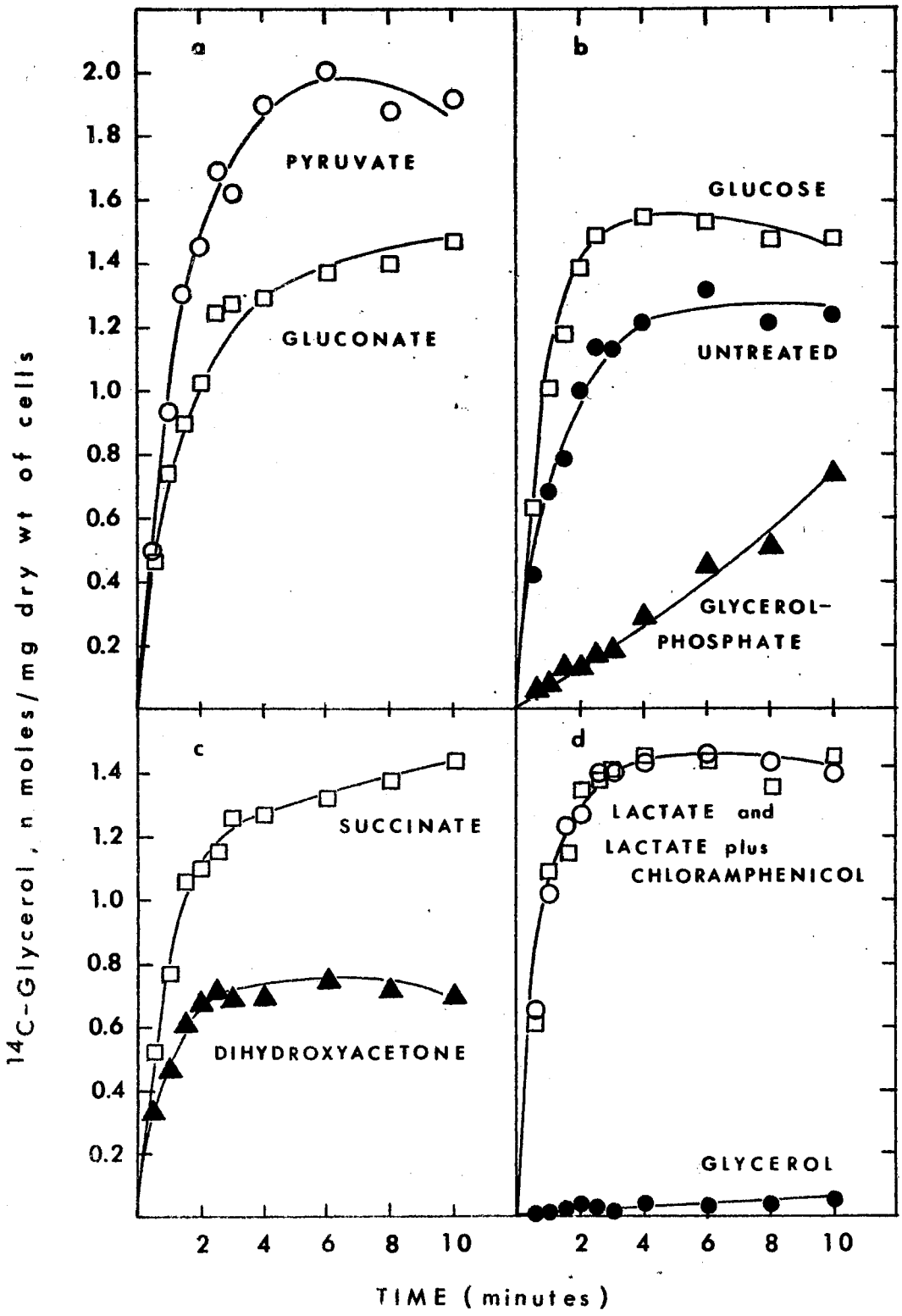
The majority of compounds tested either produced no effect or caused a stimulation of glycerol uptake. This latter phenomenon produced by sodium lactate, sodium pyruvate, glucose, disodium succinate, potassium gluconate, 1,2-propanediol, 1,3-propanediol, 1,2,4-butanetriol, and ethanol will be referred to as the "lactate effect".

The only two compounds to produce significant inhibition were DL-$\alpha$-glycerophosphate and dihydroxyacetone (closed triangles). The closed-triangle curve in Figure 12b indicated that the initial inhibition by DL-$\alpha$-glycerophosphate was being overcome with time. Soon after this set of experiments was run, Berman and Lin (1971) reported that commercial DL-$\alpha$-glycerophosphate is contaminated with glycerol. This could explain the apparent inhibition of transport since the specific activity of the glycerol would be decreased. However, $\beta$-glycerophosphate produced no inhibitory effect in the same type of experiment (results not shown).

Figure 12. Transport of Glycerol-U-$^{14}C$ by Induced PA-$1_L$ Preloaded With Various Nonradioactive Compounds.

PA-$1_L$ cells were grown in 0.5 per cent sodium lactate minimal medium A and induced in 0.5 per cent glycerol minimal medium A, after which they were harvested, washed, and prepared for assay of transport activity following the procedure described in Materials and Methods. When effects of "cold" compounds were tested, these were added to 0.01 M (preloading the cells) at one minute before zero time. Names of the nonradioactive substances added are shown beside the appropriate curve.

a - d. Two controls were run (●): untreated PA-$1_L$ (b) and PA-$1_L$ preloaded with 0.01 M glycerol, an expected competitor (d). Curves with added compounds appearing to produce an inhibitory effect are represented by closed triangles (▲) whereas those with added compounds demonstrating a stimulatory effect are designated by open circles (o) and open squares (□).


a
b
c
d
PYRUVATE
GLUCONATE
GLUCOSE
UNTREATED
GLYCEROL-
PHOSPHATE
SUCCINATE
DIHYDROXYACETONE
LACTATE and
LACTATE plus
CHLORAMPHENICOL
GLYCEROL
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
TIME ( minutes )
14C-Glycerol, n moles/mg dry wt of cells

Upon addition of dihydroxyacetone (DHA) the total glycerol uptake was reduced by about one-half. Only the 30-second value remained approximately normal. One must assume that metabolism was occurring in these wild-type cells. Thus, the appearance of the latter phase of the $^{14}C$-glycerol uptake curves could result from release of a $^{14}C$-intermediate at the same rate or at a rate faster than uptake was occurring. The most probable intermediate would be $^{14}CO_2$. If this were the case, the inhibitory effect observed upon addition of DHA could actually be caused by an increased production of $^{14}CO_2$, whereas the stimulative effects observed after addition of lactate, pyruvate, gluconate, succinate, or glucose could be due to a reduction in $^{14}CO_2$ released. It is noteworthy that the addition of 50 µg/ml of chloramphenicol to the cell suspension before the transport assay in no way affected the stimulative "lactate effect".

## Effect of $^{14}CO_2$ Production During Measurement of Transport

A search of the transport literature indicates that researchers generally consider transport plateaus as indicative of a steady-state, i.e., an equilibration between influx and outflow of the substrate. Of course, the majority of studies involve nonmetabolizing mutants. However, there is no mention of possible release of $^{14}C$-intermediates and what the result might be.

Since release of $^{14}CO_2$ seemed a real possibility in this study, a modified $CO_2$-trapping technique was developed and used to measure the amount of $^{14}CO_2$, if any, that was released during the transport assay. The method is described in Materials and Methods. Again, in this type of experiment, when a decrease in $^{14}CO_2$ release was expected (as for

lactate) the untreated control was run last. The untreated control was run first in the case of dihydroxyacetone. Since the two untreated control curves were identical only one is shown in Figure 13.

At thirty seconds into transport no appreciable amounts of $^{14}CO_2$ were released except upon addition of dihydroxyacetone. It was quite evident, however, that this particular compound greatly stimulated the production of $^{14}CO_2$ from $^{14}C$-glycerol throughout the assay period. The addition of sodium lactate caused a slight increase in $^{14}CO_2$ production for the first 2 min, but then this level declined in relation to that of the untreated control until at 10 min there was a 20 per cent deficit.

Analysis of these data is complicated. However, it does seem that the effect of DHA on transport could be explained by excessive $^{14}CO_2$ production. The interpretation of the "lactate effect" is more difficult. The increase in glycerol transport was apparent at 30 seconds but at that time the level of $^{14}CO_2$ released was insignificant. Then, for the next 90 seconds both $^{14}CO_2$ production and $^{14}C$ uptake were increased. The only portion of the stimulated transport curve which could be explained by loss of $^{14}CO_2$ was the plateau. Thus, there may or may not be a direct relationship between $CO_2$ release and appearance of the glycerol transport curve upon addition of such substances as lactate, glucose, and pyruvate. Several other explanations are possible.

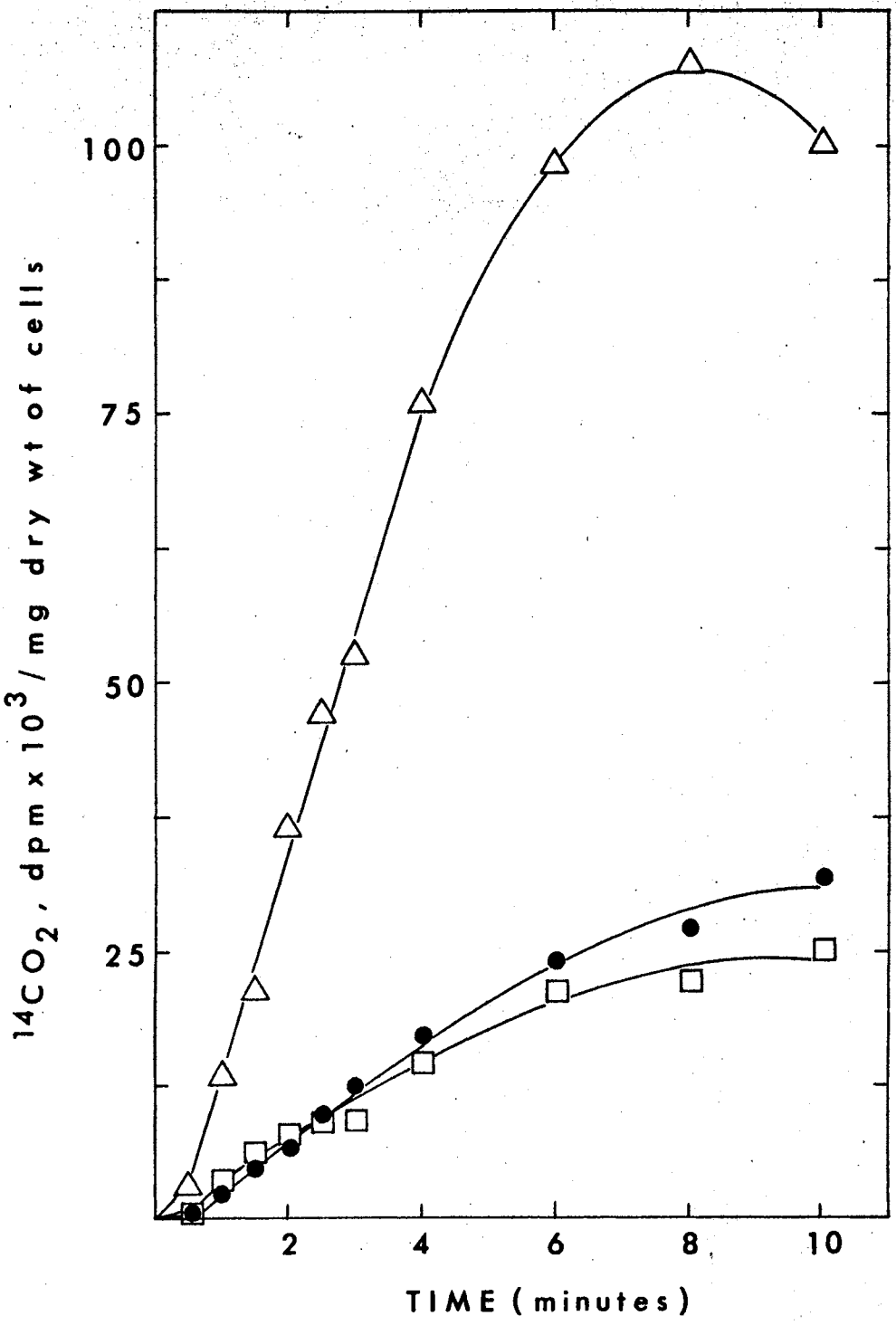
Analysis of the "Lactate Effect"

Sodium lactate, glucose, etc., since they are readily metabolized carbon sources for P. aeruginosa, could serve as second substrates

Figure 13. Effect of Sodium Lactate and Dihydroxyacetone on Release of $^{14}CO_2$ by PA-$1_L$ Supplied With Glycerol-U-$^{14}C$.

Glycerol-grown PA-$1_L$ cells were prepared for measurement of $^{14}CO_2$ accumulation according to the procedure described in Materials and Methods.

$^{14}CO_2$ accumulation was measured on untreated PA-$1_L$ (control ●); PA-$1_L$ to which 0.01 M sodium lactate was added at one minute before zero time (□); and PA-$1_L$ to which 0.01 M dihydroxyacetone was added at one minute before zero time (△).


100
75
50
25
2
4
6
8
10
TIME ( minutes )
$^{14}CO_2$ , dpm x $10^3$ / mg dry wt of cells

providing additional energy for metabolism of glycerol. The amounts and types of stimulation could possibly be explained by considering the ease of entry of the substrate and amount of ATP produced. The glycerol kinase reaction requires ATP, and, if this capturing of glycerol as glycerophosphate were the rate-limiting step, the over-all appearance would be increased uptake of $^{14}$C-glycerol and increased $^{14}CO_2$ production until the energy is depleted. Examination of this possibility was made by observing glycerol transport of uninduced wild-type cells (glucose-grown) after addition of lactate, pyruvate, and glucose (Table X).

Previously-shown enzyme data (Table VI) had demonstrated that PA-$1_L$ grown on glucose had insignificant levels of glycerol kinase and L-$\alpha$-glycerophosphate dehydrogenase. Nevertheless, the normally-low level of $^{14}$C-glycerol uptake by freshly-prepared PA-$1_L$ was doubled by the end of six minutes upon addition of either lactate, glucose, or pyruvate. It should be noted that, in all cases, the same effects were observed at 30 seconds but the values were quite low. The four or six-minute measurements appeared more explicit.

After harvesting, washing, and preparing the fresh cells for uptake, the remaining unused suspension was placed on the 37 C shaker for 2 additional hours. During this time the cells were without $MgSO_4 \cdot 7H_2O$ and a carbon and energy source. These starved wild-type cells were harvested, washed, resuspended to 40 per cent transmittance, and also examined for levels of glycerol transport after addition of pyruvate or glucose. Again, a doubling of the untreated rate was seen at the end of six minutes.

TABLE X

EFFECT OF ADDITION OF SODIUM PYRUVATE AND GLUCOSE ON $^{14}$C-GLYCEROL TRANSPORT BY UNINDUCED PA-$1_L$ AND GA-73 AND BY INDUCED MUTANTS

| Strain | Substrate/Inducer | Treatment | nmoles $^{14}$C-Glycerol Taken Up | Time Minutes |
|---|---|---|---|---|
| PA-$1_L$ | Glucose | Freshly Prepared No Additions | 0.23 | 6 |
| PA-$1_L$ | Glucose | Freshly Prepared Lactate Added | 0.51 | 6 |
| PA-$1_L$ | Glucose | Starved 2 hr No Additions | 0.24 | 6 |
| PA-$1_L$ | Glucose | Starved 2 hr Pyruvate Added | 0.50 | 6 |
| PA-$1_L$ | Glucose | Starved 2 hr Glucose Added | 0.53 | 6 |
| PA-$1_L$ | Succinate | Starved 2 hr No Additions | 0.31 | 6 |
| PA-$1_L$ | Succinate | Starved 2 hr Pyruvate Added | 0.31 | 6 |
| PA-$1_L$ | Succinate | Starved 2 hr Glucose Added | 0.23 | 6 |
| GA-73 | Glucose | Freshly Prepared No Additions | 1.57 | 6 |
| GA-73 | Glucose | Freshly Prepared Pyruvate Added | 2.15 | 6 |
| GA-73 | Glucose | Freshly Prepared Glucose Added | 2.31 | 6 |
| AH-8 | Glucose/Glycerol | Freshly Prepared No Additions | 1.01 | 4 |
| AH-8 | Glucose/Glycerol | Freshly Prepared Pyruvate Added | 1.71 | 4 |
| AH-8 | Glucose/Glycerol | Freshly Prepared Glucose Added | 1.60 | 4 |
| PA-1-623 | Glucose + Methionine | Freshly Prepared No Additions | 0.19 | 6 |
| PA-1-623 | Glucose + Methionine | Freshly Prepared Methionine Added | 0.18 | 6 |
| PA-1-623 | Glucose/Glycerol | Freshly Prepared No Additions | 0.21 | 6 |
| PA-1-623 | Glucose/Glycerol | Freshly Prepared Pyruvate Added | 0.18 | 6 |
| PA-1-623 | Glucose/Glycerol | Freshly Prepared Glucose Added | 0.19 | 6 |

Abbreviations are as follows: Glucose + Methionine, grown in the presence of both 0.5 per cent glucose and 35 µg/ml methionine; Glucose/Glycerol, glucose-grown glycerol-induced.

Earlier (Table VI), wild-type cells grown on succinate were also shown to have insignificant levels of the glycerol degradative enzymes; however, the transport of glycerol by these cells was unaffected by addition of glucose or pyruvate.

$^{14}$C-glycerol transport by GA-73, earlier characterized as a glycerol kinaseless mutant, also responded to addition of pyruvate and glucose by exhibiting stimulation. In this instance, the rate was not doubled but was noticeably increased.

The four-minute sampling time was chosen for AH-8 cells in order to avoid the latter phase of its glycerol transport during which $^{14}$C is lost. Here again, addition of either pyruvate or glucose produced a stimulation in uptake.

PA-1-623 which was previously shown to have glycerol kinase activity whether it was grown on glucose or induced for four hours on glycerol, was tested under two sets of conditions. Since it is a methionine auxotroph, one measurement of uptake was made on fresh cells plus methionine. The second set of transport measurements was made on induced PA-1-623 cells treated with pyruvate or glucose one minute before zero time. The uptake curves were unaffected in both instances.

In summary, then, metabolic effects do not appear to explain the "lactate effect." Not only do nonmetabolizing PA-$1_L$ cells continue to display stimulation throughout 6 minutes of transport, but so do induced cells lacking glycerol kinase (GA-73) and L-$\alpha$-glycerophosphate dehydrogenase (AH-8), respectively. Koch and Coffman (1970) have stated that enzymes and not diffusion are rate-limiting in cryptic cells unless a significant number of enzyme molecules is present. This level could be exceeded in PA-1-623 since it is constitutive for

glycerol kinase. Then, even if the kinase activity were increased the diffusion would remain constant. However, it is doubtful that this explains the behavior of succinate-grown PA-$1_L$ since the enzyme levels involved here are lower than those of glucose-grown PA-$1_L$. Therefore, the stimulatory effect of lactate, etc., may be more closely related to the transport mechanism itself, perhaps to binding.

Entrance Counterflow

Winkler and Wilson (1966) have proposed that energy coupling actually results in a marked reduction in affinity of the membrane carrier for its substrate at the inner surface of the membrane. Thus, energy, in some form, releases substrate from carrier inside the cell and eliminates any significant efflux. Energy inhibitors, then, do not affect influx but, rather, increase efflux to such an extent that transport appears minimal. Extending this hypothesis, Wong and Wilson (1970) postulated that cells preloaded with competitive inhibitors would have the one-way movement of substrate from one side of the membrane blocked while movement in the opposite direction would proceed. The result would be movement of substrate against a gradient and would appear as a stimulation of substrate transport. Support for this hypothesis was provided by experiments similar to the previously-mentioned competition experiments except that energy-poisoned, uninduced cells were used, preloading them with competitor and then, at zero time, adding $^{14}$C-TMG and measuring transport. Stimulation of uptake similar to that observed upon addition of lactate, glucose, or pyruvate to glucose-grown PA-$1_L$ or GA-73 and AH-8 was observed. $^{14}$C-TMG transport was also stimulated in energy-poisoned, induced

E. coli preloaded with various competitors.

Since sodium lactate, glucose, disodium succinate, and potassium gluconate had already proven to be effective competitors for glycerol binding by glucose-grown cells (Tables VII and VIII), this Wong and Wilson hypothesis seemed to provide a feasible explanation. Even the stimulation evident in glycerol transport by lactate-grown, glycerol-induced PA-$1_L$, after addition of any of the previously-mentioned substances, could be interpreted in this manner if lactate were inducing a general glycerol binder (as glucose does), this being the affected portion. However, as was noted earlier, glycerol-grown cells also displayed the "lactate effect" yet neither lactate, pyruvate, succinate, nor gluconate effectively inhibited glycerol binding in these cells.

## Exit Counterflow

Wong and Wilson described a reciprocal experiment which would further support their hypothesis. Energy-poisoned, induced cells were preloaded with radioactive substrate, after which a "cold" competitor was added. These investigators observed an immediate net loss of $^{14}C$, the amount being directly proportional to the efficiency of the competitor.

PA-$1_L$ cells were treated similarly except that cells were not energy-poisoned. Transport by lactate-grown, glycerol-induced wild-type cells was measured without additions for 120 seconds (samples were taken at 30-second intervals). At 135 seconds, 10 mmole quantities of sodium lactate, glucose, D-fructose, potassium gluconate, DL-$\alpha$-glycerophosphate, or glycerol were added. Sampling was continued up

to 10 minutes in some cases. Results are presented in Figures 14a-14d.

The majority of the curves displayed by Wong and Wilson looked like that for DL-α-glycerophosphate. They interpreted the sharp drop and recovery to indicate that the substrate was competing but not being metabolized. On the other hand, a curve like that for glycerol was indicative of a metabolizable competitor. Since the rates of glycerol transport after the addition of lactate or glucose continued for a longer period of time than normal, stimulation of uptake still seemed to be the effect. In comparison, addition of fructose or gluconate during transport appeared to produce no effect at all. Therefore, the "lactate effect" does not seem to be due to competition either. However, there was still the possibility that a relationship between energy-coupling and binding existed and that these stimulatory compounds provided additional energy through their own metabolism. In this case, an energy requirement for $^{14}$C-glycerol transport should be detectable by three procedures, two involving use of common energy inhibitors.

## Determination of Energy-Coupling in Glycerol-U-$^{14}$C Transport

### Preincubation With Energy Inhibitors

In the first type of experiment, 24.9 ml of PA-$1_L$ cell suspension at 45 per cent transmittance were placed into a 250-ml Erlenmeyer flask with the specified amount of the desired energy inhibitor and allowed to incubate 30 minutes at 37 C in a reciprocal shaking water bath. At zero time $^{14}$C-glycerol was added and samples were taken as for any other transport measurement. Results are plotted in Figures 15a-15d.

Figure 14. Effect of Adding Various Nonradioactive Compounds During Assay of Glycerol-U-$^{14}C$ Transport.

$^{14}C$-glycerol transport by lactate-grown, glycerol-induced PA-$1_L$ was measured normally, sampling at 30-second intervals for 120 seconds. At 135 seconds after zero time, various "cold" compounds were added to the suspension to 0.01 M, after which sampling was continued for several minutes. The arrows indicate the points at which nonradioactive substances were added.

a. Glycerol transport before and after addition of 0.01 M glucose (△).

b. Glycerol transport before and after addition of 0.01 M sodium lactate (○) or DL-α-glycerophosphate (●).

c. Glycerol transport before and after addition of 0.01 M D-fructose (□) or 0.01 M glycerol (▲).

d. Glycerol transport before and after addition of 0.01 M potassium gluconate (○).

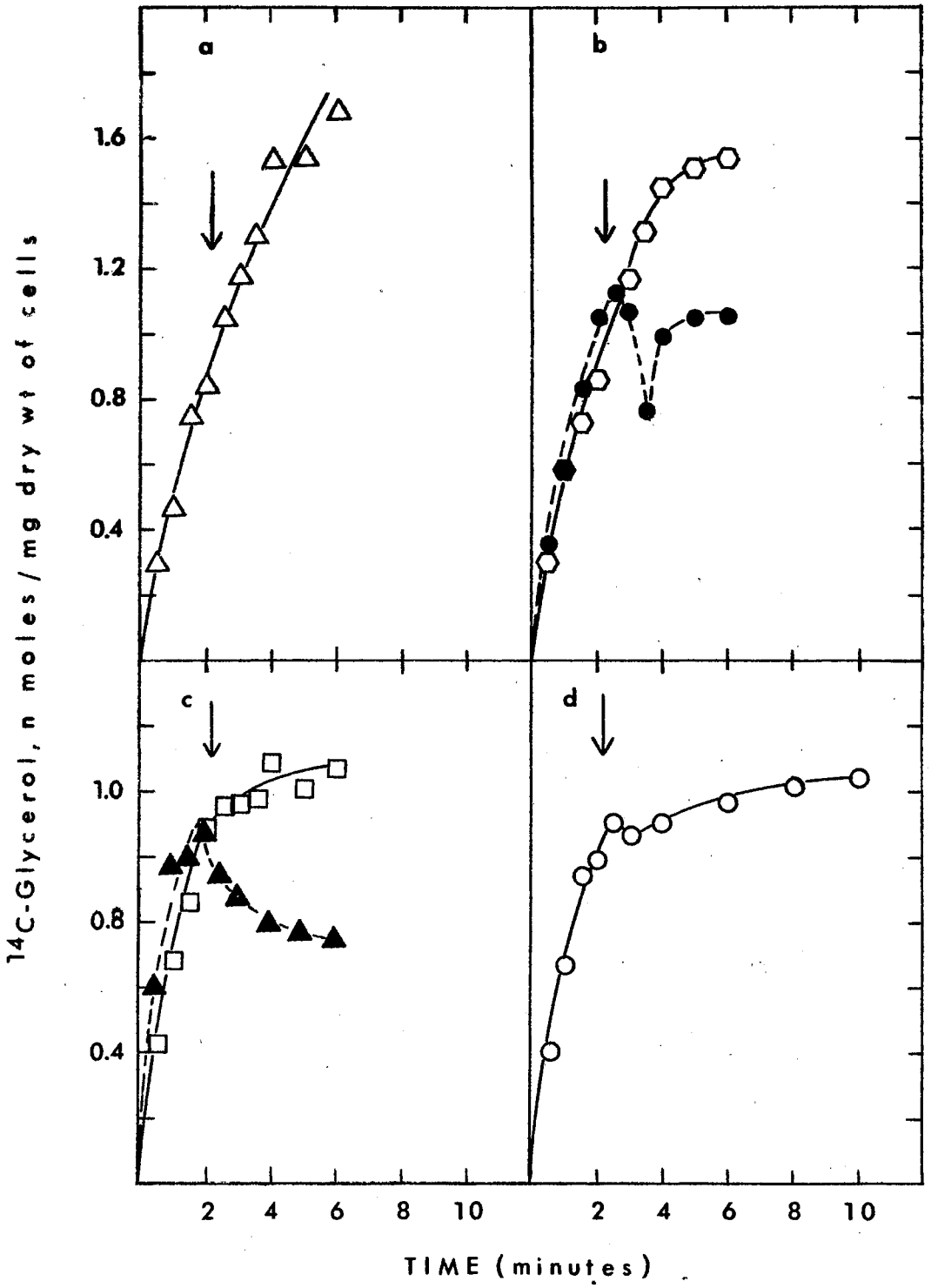

a
b
c
d
1.6
1.2
0.8
0.4
1.0
0.8
0.4
2
4
6
8
10
2
4
6
8
10
TIME (minutes)
$^{14}$C-Glycerol, n moles / mg dry wt of cells

Figure 15. Transport of Glycerol-U-$^{14}$C by Induced PA-$1_L$ Pre-treated With Known Energy Inhibitors.

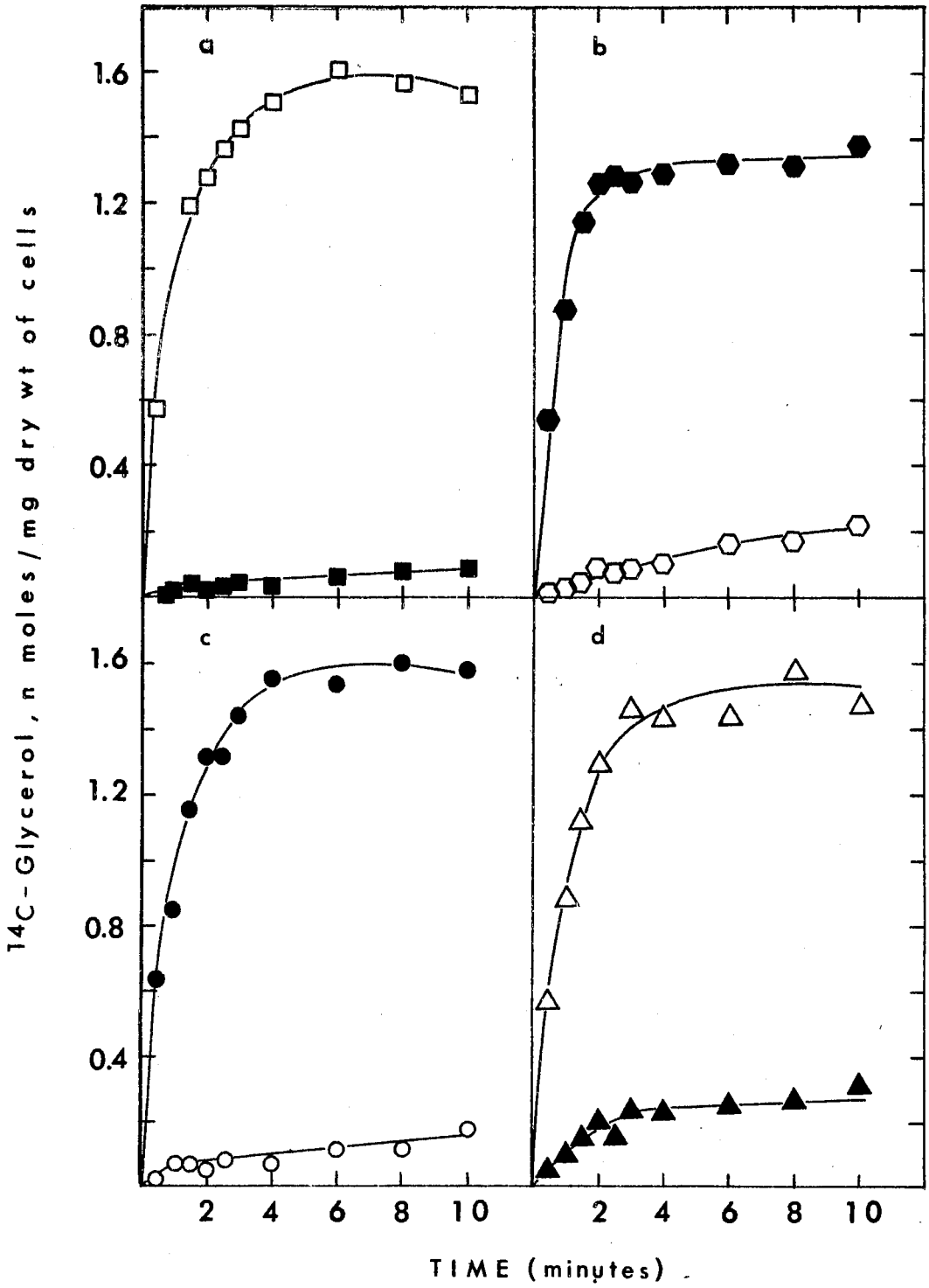
Glycerol-grown PA-$1_L$ cells were prepared for transport assay as previously described in Materials and Methods. Known energy inhibitors were added to the 45 per cent transmittance cell suspension, to the concentrations specified below, at 30 minutes before zero time (pre-treating the cells). At zero time $^{14}$C-glycerol was added with samples being removed as usual for measurement of transport.

a. Glycerol-U-$^{14}$C transport by PA-$1_L$ pre-treated with $10^{-5}$ M oligomycin (□) or 0.03 M sodium azide (■).

b. Glycerol-U-$^{14}$C transport by untreated PA-$1_L$ control (●) and by PA-$1_L$ pre-treated with 0.03 M sodium cyanide (○).

c. Glycerol-U-$^{14}$C transport by PA-$1_L$ pre-treated with absolute ethanol (control for uptakes measured in the presence of oligomycin and antimycin A, ●), or 0.01 M 2,4-dinitrophenol (o).

d. Glycerol-U-$^{14}$C transport by PA-$1_L$ pre-treated with $10^{-5}$ M antimycin A (△) or 0.01 M sodium cyanide (▲).


a
b
c
d
1.6
1.2
0.8
0.4
2
4
6
8
10
$^{14}$C-Glycerol, n moles/mg dry wt of cells
TIME (minutes)

Upon referring back to Tables VII and VIII, it was noted that both sodium cyanide and sodium azide inhibited binding of glycerol by glycerol-grown cells. It was not surprising, then, to see that these compounds also inhibited glycerol transport. In addition, the curves in Figures 15b and 15d suggested that addition of a greater quantity of sodium cyanide reduced the initial curvature expressed. 2,4-Dinitrophenol, at 10 mM, had no effect on glycerol binding by glycerol-grown cells but did inhibit glycerol transport. The slight initial curvature, according to Winkler and Wilson (1966) is indicative of the proportion of facilitated diffusion present. Several investigators (Whittam, et al., 1964; Kaback and Stadtman, 1968; and Barnes and Kaback, 1970) have noted the detrimental effects of electron transport inhibitors such as oligomycin or antimycin A on active transport by membrane preparations or erythrocytes. The two inhibitors had to be dissolved in absolute ethanol before use. Therefore, the control curve for these inhibitors (closed circles, 15c) depicts glycerol transport by PA-$1_L$ cells preincubated 30 minutes with 0.1 ml of absolute ethanol. Oligomycin appeared to cause only slight reduction of glycerol transport whereas antimycin A produced no effect at all. (During preparation of this thesis a report appeared [Ishaque, et al., 1971] in which it was shown that high concentrations of oligomycin and antimycin A are required to inhibit electron transport in P. saccharophila. This may explain the lack of effect noted in the present study.)

## Addition of Energy Inhibitors to Preloaded PA-$1_L$ Cells

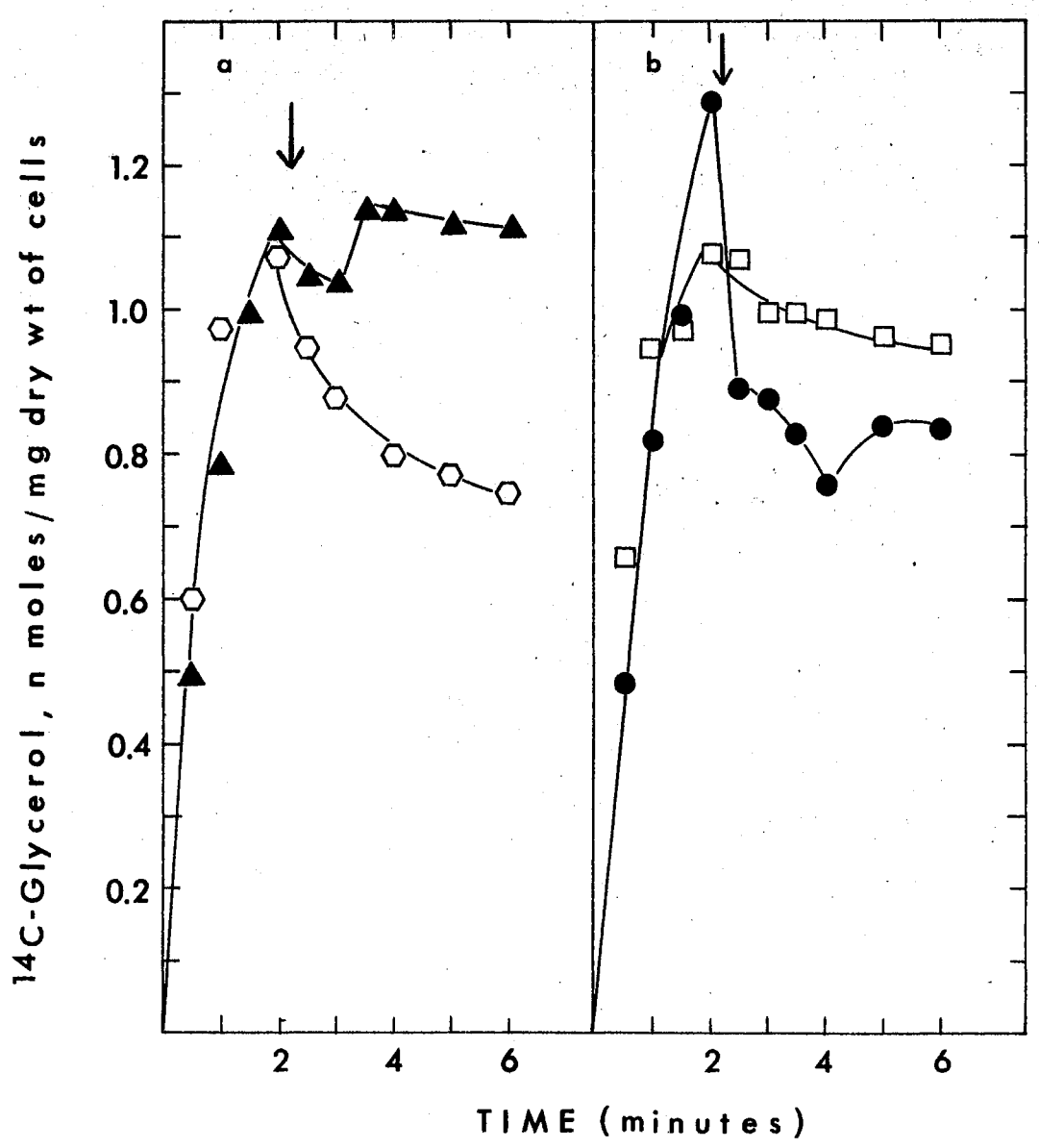
Figure 16 illustrates the effects of preloading PA-$1_L$ cells with $^{14}$C-glycerol and adding inhibitors at 135 seconds into transport.

Figure 16. Effect of Adding Energy-Inhibiting Compounds During Assay of Glycerol-U-$^{14}$C Transport.

$^{14}$C-glycerol transport by lactate-grown, glycerol-induced PA-$1_L$ was measured normally, sampling at 30-second intervals for 120 seconds. At 135 seconds after zero time, known energy-inhibiting compounds were added to the suspension (concentrations specified below), after which sampling was continued for several minutes. The arrows indicate points at which inhibitors were added.

a. Glycerol-U-$^{14}$C transport before and after addition of 0.01 M glycerol (control, ○) or 0.03 M sodium cyanide (▲).

b. Glycerol-U-$^{14}$C transport before and after addition of 0.03 M sodium azide (●) or 0.01 M 2,4-dinitrophenol (□).


a
b
1.2
1.0
0.8
0.6
0.4
0.2
2
4
6
2
4
6
14C-Glycerol, n moles / mg dry wt of cells
TIME (minutes)

The addition of "cold" glycerol at 10 mM (Figure 16a) was included in this set as a control curve. All inhibitors produced a net loss of $^{14}C$. Sodium cyanide caused a slight initial loss which was quickly recovered (the curve was similar to that of a nonmetabolizable partial competitor). 2,4-Dinitrophenol produced a steady loss of $^{14}C$ which, by six minutes, approached 17 per cent of the total $^{14}C$-glycerol taken up. Sodium azide produced the most dramatic effect with a 30 per cent loss occurring within 15 seconds after the addition, reaching a total loss of 44 per cent at four minutes. This set of experiments was carried out on both lactate-grown, glycerol-induced cells and glycerol-grown cells. It is noteworthy that over-all effects and shapes of curves were identical in both sets of cells. However, the amounts of losses by glycerol-grown cells were lower, e.g., 20 per cent at the most upon addition of azide.

The 44 per cent $^{14}C$ loss was certainly significant if, in fact, it represented a net loss of $^{14}C$-glycerol. In order to identify the released compound(s), a similar experiment was run, chromatographing supernatants. Lactate-grown, glycerol-induced PA-$1_L$ cells were prepared for transport assay as normal. Fifty milliliters of the cell suspension (45 per cent transmittance) were placed into a 500-ml Erlenmeyer flask. At zero time, 0.5 ml of glycerol-U-$^{14}C$ was added to the flask. The first 5.0-ml sample was removed and filtered at 120 seconds into transport, being washed immediately with two 1-ml portions of minimal medium B. Sodium azide (30 mM) was added to the flask at 135 seconds. Additional 5.0-ml samples were removed and treated as above at 180, 210, 240, and 270 seconds into transport. Individual filtrates, including wash solutions, were collected in 20 mm tubes, the contents

being frozen and lyophilized immediately. The dried solids remaining in each tube were resuspended in 1.0 ml of glass-distilled water and lyophilized again before their final suspension in 0.3 ml of glass-distilled water.

In order to determine whether sodium azide caused a large-scale release of cell components, a check was made on the filtrates for the presence of nucleotides and/or proteins. A 0.1 ml aliquot of the resuspended filtrate was placed into a cuvette, diluted with 2.9 ml of glass-distilled water and scanned (against a glass-distilled water blank) through the UV spectra on a Cary 14 recording spectrophotometer. The remaining 0.2 ml of each filtrate was placed in a band on separate 60-cm paper strips (Whatman # 4) which had been prewashed with a distilled water, pyridine, glacial acetic acid solution (80:15:5 v/v/v). Ten microliters of 1 per cent solutions of the nonradioactive standards (glycerol, DL-$\alpha$-glycerophosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, DL-glyceric acid and dihydroxyacetone) and 20 $\mu$l of glycerol-U-$^{14}$C were also spotted separately. All strips were chromatographed in a solution of isopropyl alcohol, pyridine, glacial acetic acid, and distilled water in the volume ratio 8:8:1:4 (Gordon, et al., 1956) for 10 hours. The strips were dried and counted on a Packard Model 7201 radiochromatogram scanner, after which the spots were developed by a procedure described by Gordon, et al. (1956). This method consisted of first dipping each strip through a periodic acid-acetone solution, then drying for 3 to 4 minutes, and finally, dipping through a benzidine-acetone solution. White, yellow, or brown spots appeared on the dark blue background of the paper. The distances traveled by glycerol-U-$^{14}$C and the "cold" glycerol were the same.

All of the filtrates displayed absorption between 250 nm and 260 nm. However, the peak decreased with time and in no way correlated with the $^{14}C$ release described in Table XI. In Table XI, peak # 2 was assumed to represent glycerol. It was present, as expected, before the azide was added and increased to its maximum at 210 seconds into transport. Peak # 1, thought to represent either D-glyceraldehyde or dihydroxyacetone phosphate, appeared only after the azide addition but increased somewhat in each successive sample. Peak # 3 did not appear until 240 seconds into transport: it possibly represented dihydroxyacetone. Interestingly, none of the filtrates contained a radioactive spot with an $R_G$ corresponding to that of DL-α-glycerophosphate.

## Boiling-Water Extraction of PA-$1_L$ and GA-73 Cells Exposed to Glycerol-U-$^{14}C$

According to Christensen (1962), the most reliable method of proving the presence of active transport is to demonstrate accumulation of the unchanged substrate, against a gradient, inside the cell. This requires the use of either a nonmetabolizable substrate or a nonmetabolizing mutant. In this study GA-73 was chosen, as the latter, for analysis of $^{14}C$-glycerol accumulation, with PA-$1_L$ serving as a control.

PA-$1_L$ and GA-73 cells were grown in glucose-glycerol minimal medium A, harvested and washed as for a transport assay, and resuspended in minimal medium B to 5 per cent transmittance. Each strain was treated separately, with 2.5 ml of cell suspension placed into each of five 25-ml Erlenmeyer flasks. Glycerol-U-$^{14}C$ (0.2 ml) was added to each flask at zero time. At 30, 60, 90, and 120 seconds a 2.0-ml sample was removed from flasks # 1 through # 5, respectively, filtered,

TABLE XI

RADIOACTIVE SPOTS IN CHROMATOGRAPHED SUPERNATANTS FROM PA-$1_L$ TREATED AT 135 SECONDS WITH 30 mM SODIUM AZIDE

| Time (Seconds) | Peak Number 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| | $R_G$ | $R_H$ | $R_G$ | $R_H$ | $R_G$ | $R_H$ |
| 120 | - | - | 1.00 | 10 | - | - |
| 180 | 0.80 | 5 | 1.00 | 35 | - | - |
| 210 | 0.86 | 20 | 1.00 | 38 | - | - |
| 240 | 0.84 | 21 | 0.98 | 28 | 1.09 | 29 |
| 270 | 0.80 | 24 | 0.94 | 27 | 1.08 | 19 |

Abbreviations are as follows: $R_G$, distance of peak relative to the distance traveled by $^{14}$C-glycerol; $R_H$, (arbitrary unit) relative height of peak as measured on strip-counting paper on a scale of 1 to 100.

Relative distances traveled are as follows: glycerol, 1.00; D,L-α-glycerol phosphate, 0.69; dihydroxyacetone, 1.05; dihydroxyacetone phosphate, 0.85; DL-glyceraldehyde, 0.85; DL-glyceric acid, 0.49.

and washed immediately with two 1.0-ml portions of minimal medium B. The filters were immediately removed and placed into a 125-ml Erlenmeyer flask. After the addition of 4 ml of boiling glass-distilled water, the flask was placed into a boiling water bath for 5 minutes. Intermittent swirling was used to remove as many cells as possible from the filter. The suspension prepared from cells washed from the filter was centrifuged in the cold at 10,000 X g for 10 minutes, after which the supernatant was poured off and saved and the pellet was resuspended in 1.0 ml of glass-distilled water. A 0.1 ml aliquot of each of the two fractions was placed into 10 ml of scintillation fluid A and counted (labeled Water Extract and Pellet in Table XII). Forty microliters of the remaining water extracts were spotted in the center of two DEAE cellulose paper discs (23 mm) and dried. One filter (disc) was washed with distilled water according to the procedure of Newsholme et al. (1967) while the second served as an untreated control. The first 2.0 ml of the distilled water wash were collected, and 1.0 ml was transferred into 9.0 ml of Aquasol scintillation fluid, and counted (labeled Wash in Table XII). The washed filter and the control filter (Table XII) were placed into scintillation vials with 10.0 ml of Aquasol and counted. Ten microliters of glycerol-U-$^{14}C$ were also spotted on two DEAE cellulose discs and treated as described above as a control to determine that the washing procedure was efficient in removing glycerol from the filter (Table XII).

The water extracts contained the majority of the radioactivity, indicating that a large portion had not yet entered the nonsoluble cell components. However, most of the $^{14}C$ in the extracts remained with the filters in both cases, although there was an apparent difference

TABLE XII

DISTRIBUTION OF $^{14}C$ FROM GLYCEROL-U-$^{14}C$ IN BOILING-WATER EXTRACTS OF PA-$1_L$ AND GA-73

| Sample Description | Mutant GA-73 | | Wild-Type (PA-$1_L$) | |
|---|---|---|---|---|
| | Total dpm | Per Cent of Control | Total dpm | Per Cent of Control |
| 30-sec. Water Extract | 161010 | | 405930 | |
| 30-sec. Pellet | 12890 | | 25790 | |
| 30-sec. Control Filter | 2359 | - | 5492 | - |
| 30-sec. Washed Filter | 2348 | 99.5 | 3315 | 60.4 |
| 30-sec. Wash | 109 | | 1952 | |
| 60-sec. Water Extract | 180960 | | 361110 | |
| 60-sec. Pellet | 11920 | | 45700 | |
| 60-sec. Control Filter | 2906 | - | 4236 | - |
| 60-sec. Washed Filter | 2242 | 77.2 | 3263 | 77.0 |
| 60-sec. Wash | 518 | | 1352 | |
| 90-sec. Water Extract | 178590 | | 373320 | |
| 90-sec. Pellet | 22260 | | 50240 | |
| 90-sec. Control Filter | 2553 | - | 5193 | - |
| 90-sec. Washed Filter | 1640 | 64.2 | 4133 | 79.6 |
| 90-sec. Wash | 506 | | 982 | |
| 120-sec. Water Extract | 226710 | | 330660 | |
| 120-sec. Pellet | 27780 | | 72690 | |
| 120-sec. Control Filter | 3207 | - | 3927 | - |
| 120-sec. Washed Filter | 1512 | 47.2 | 3774 | 96.1 |
| 120-sec. Wash | 994 | | 650 | |
| Glycerol Control Filter | 1046 | - | | |
| Glycerol Washed Filter | 100 | | | |
| Glycerol Wash Solution | 1040 | 99.5 | | |

between PA-$1_L$ and GA-73. The 30-second washed filter retained all of the $^{14}C$ product(s) from the GA-73 water extract whereas only 60 per cent of that from the PA-$1_L$ extract was retained. With time, the amount retained from the GA-73 extract decreased while that retained from the PA-$1_L$ extract increased to 96 per cent by 2 minutes.

The remaining boiling-water extracts were lyophilized, resuspended in 0.2 ml of glass-distilled water, and band-chromatographed following the same procedures as described previously. A number of nonradioactive spots were detected but only two radioactive ones were apparent: both were located near the origin and did not correspond directly to any of the standards although peak # 2 is within the range of glyceraldehyde-3-phosphate. Variations in these materials with time are shown in Table XIII. Again, a significant difference was found between GA-73 and PA-$1_L$. Peak # 1 decreased with time in GA-73 extracts, while peak # 2 increased. The opposite effect was seen in PA-$1_L$ extracts. It may be noted that a large nonradioactive spot at a position corresponding to that of DL-α-glycerophosphate was present in all extracts.

The results of the last two experiments strongly suggested a major metabolic difference between PA-$1_L$ and GA-73, with GA-73 somehow utilizing glycerol but not growing on it. This possibility required further investigation and the pattern of $^{14}CO_2$ accumulation from glycerol-U-$^{14}C$ was analyzed. Uninduced (glucose-grown) and induced cells of PA-$1_L$ and GA-73 were assayed for $^{14}CO_2$ release during the transport period. One set of induced cells for each strain was also tested after treatment with DHA at one minute before zero time. Only the six-minute value is recorded in Table XIV. Interestingly,

TABLE XIII

CHROMATOGRAPHY OF RADIOACTIVE MATERIALS FROM EXTRACTS OF GA-73 and PA-$1_{S+L}$

| Time (Seconds) | GA-73 Peak Number 1 $R_G$ | GA-73 Peak Number 1 $R_H$ | GA-73 Peak Number 2 $R_G$ | GA-73 Peak Number 2 $R_H$ | $PA\text{-}1_{S+L}$ Peak Number 1 $R_G$ | $PA\text{-}1_{S+L}$ Peak Number 1 $R_H$ | $PA\text{-}1_{S+L}$ Peak Number 2 $R_G$ | $PA\text{-}1_{S+L}$ Peak Number 2 $R_H$ |
|---|---|---|---|---|---|---|---|---|
| 30 | 0.02 | 23 | 0.21 | 12 | 0.03 | 58 | 0.19 | 28 |
| 60 | 0.02 | 25 | 0.23 | 16 | 0.02 | 55 | 0.19 | 32 |
| 90 | 0.01 | 3 | 0.23 | 38 | 0.03 | 94 | 0.19 | 21 |
| 120 | 0.01 | 6 | 0.21 | 62 | 0.02 | 120 | 0.21 | 9 |

Abbreviations are as follows: $R_G$, distance of peak relative to the distance traveled by $^{14}C$-glycerol; $R_H$ (arbitrary units) height of peak as measured on strip-counting paper on a scale of 1 to 100.

Relative distances traveled are as follows: glycerol, 1.00; DL-α-glycerophosphate, 0.69; dihydroxyacetone, 1.05; dihydroxyacetone phosphate, 0.85; glyceraldehyde-3-phosphate, 0.28; glyceric acid, 0.49.

TABLE XIV

$^{14}CO_2$ ACCUMULATION BY UNINDUCED AND INDUCED PA-$1_L$ AND GA-73 AND INDUCED AH-8

| Strain | Growth Substrate/ Inducer | Treatment | $^{14}CO_2$ dpm/mg Dry Wt of Cells at 6 Minutes |
|---|---|---|---|
| PA-$1_L$ | Glucose | None | 6425 |
| PA-$1_L$ | Glycerol | None | 24472 |
| PA-$1_L$ | Glycerol | Addition of DHA | 98625 |
| GA-73 | Glucose | None | 17900 |
| GA-73 | Glucose/Glycerol | None | 7920 |
| GA-73 | Glucose-Glycerol | None | 30660 |
| GA-73 | Glucose-Glycerol | Addition of DHA | 10410 |
| AH-8 | Glucose-Glycerol | None | 1115 |
| AH-8 | Glucose-Glycerol | Addition of DHA | 2000 |

Abbreviations are as follows: DHA, dihydroxyacetone; Glucose/Glycerol, glucose-grown glycerol-induced; Glucose-Glycerol, grown in a medium with both glucose and glycerol.

glycerol-grown PA-$1_L$ and glucose-glycerol grown GA-73 produced approximately the same amounts of $^{14}CO_2$. Induction of glucose-grown GA-73 in glycerol, or addition of DHA to the glucose-glycerol grown cells greatly reduced this output. In contrast, uninduced PA-$1_L$ cells produced approximately one-third as much $^{14}CO_2$ as did uninduced GA-73 and addition of DHA to induced wild-type cells significantly increased the yield of $^{14}CO_2$ instead of decreasing it. The behavior of AH-8 was similar to that of PA-$1_L$ but on a reduced level.

# CHAPTER IV

## DISCUSSION

The first problem encountered in this investigation was the repeated irreversible dissociation of the large colony-forming PA-1 to the small colony variant. This phenomenon has been extensively described in early studies on P. aeruginosa with possible explanations being few (Hadley, 1927; Kramer, 1935; Gaby, 1946; Zierdt and Schmidt, 1964). Gaby stated that the transformation of large mucoid colonies to small dry colonies could be initiated by animal passage, growth in high concentrations of glycerol, or repeated transfers. He postulated that the smaller type colonies carried a phage which caused the altered morphological appearance. However, he was never able to transfer the small colony characteristics via a needle or loop. Other researchers proposed other theories but none were ever proven. Nevertheless, as a result of all the experimentation on the problem, the previously-referenced workers did establish an important point. Namely, that there were no distinguishable "biochemical" differences between the colonial types although variations in flagellar antigens and bacteriophage sensitivity were noted.

In the experiments described herein, care was always taken to assure the purity of the desired colonial type. Plate checks were made on the initial inoculations and on the resultant batch culture. With few exceptions (these being noted) experimental results were discarded

unless the batch was at least 95 per cent pure.

Initial results were in agreement with those of earlier researchers. The metabolic differences between PA-$1_L$ and PA-$1_S$ were negligible. Transport and binding activities of the variants were essentially equivalent (Figure 3a and Table I) as were glycerol kinase and L-α-glycerophosphate dehydrogenase activities (not shown). In both variants, the latter specific enzyme activities averaged between 10 and 13 nmoles/min/mg of protein. However, differences between the two types did become apparent in later experiments dealing with determination of the pH profiles for binding.

These experiments, utilizing an osmotic shock procedure, presented evidence of a structural variation between PA-$1_L$ and PA-$1_S$. PPBP from large colony type cells precipitated at or before 50 per cent $(NH_4)_2SO_4$ saturation, appeared yellowish and smoothly-packed, and was difficult to dissolve in distilled water. Furthermore, excess precipitate was formed upon addition of absolute ethanol to PPBPS. On the other hand, PPBP from small colony type cells (PA-$1_S$, GA-73, and PA-1-623) precipitated between 65 and 70 per cent $(NH_4)_2SO_4$ saturation, appeared white and fluffy, and was easily dissolved in water. The latter precipitates did contain a slight amount of the previously-described yellowish sediment, but it was insignificant in relation to that from large colony type cells. Since Leive (1965d) had reported that as much as 50 per cent of a cell's lipopolysaccharide could be released by treatment with EDTA alone, this compound had to be considered as a possible contaminant in shock fluid. Its presence was detected by a modification of the LPS assay procedure used by Leive (1965d), and, as suspected, the PPBPS from large colony type cells contained close to three

times more LPS than that from PA-$1_S$, etc. Therefore, either the large colony variants contain a greater amount of LPS or the LPS of these variant types is more easily removed by the osmotic shock procedure.

This LPS difference did not seem to cause differences in transport, enzyme, or shock fluid binding activities of PA-$1_L$ and PA-$1_S$ (Figure 3a and Table I); however, pH binding profile determinations were found to be different. Peak shifts were apparent from over-all profile comparisons of PA-$1_L$ and AH-8 with PA-$1_S$ and GA-73. Since the pH binding profile data resulted from a procedure normally used on enzymes, there were no previous binding interpretations available to help explain the results. However, some investigators have reported data of specific interest. Tris-EDTA treatment of P. aeruginosa has been shown to release a protein-lipopolysaccharide complex from cell walls (Cox and Eagon, 1968; Rogers, et al., 1969) and transport of aspartate or glutamate by osmotically shocked Staph. aureus cells was reported to be stimulated by addition of an isolated lipid component from the shock fluid (Gale and Folkes, 1967; Gale and Llewellin, 1970). Also, Nikaido (1962) proposed that the $\beta$-galactoside permease of E. coli was actually complexed with phospholipid, this whole complex functioning as the membrane carrier. He has suggested that chemical changes in the phospholipid moiety could initiate conformational changes in the protein moiety and, in this way, accomplish membrane passage by the substrate. In view of these proposed interrelationships, it seems feasible to hypothesize that the presence of various amounts of a lipid component (lipopolysaccharide) could alter binding characteristics of the protein moiety, especially where pH is involved and there is a very narrow pH range at which maximum binding takes

place. Therefore, after an in-depth analysis of all collected data, the pH binding profile of the small colony variant was considered to represent the norm and that exhibited by PA-$1_L$ was considered shifted downward.

A common criticism of $^{14}C$ uptake experiments is that a false curve could be produced in various ways. For instance, a non-specific type of binding could occur on the outer cell surface or, possibly, on the membrane filter itself. Of course, such possibilities are difficult to disprove, but in order to help reduce them, two controls were run in the present investigation. Uptake of $^{14}C$-glycerol by boiled PA-$1_L$ cells was measured (Figure 3a). Although some viable cells remained after boiling ($\sim$ 0.001 per cent of the initial number), this suspension displayed an insignificant amount of uptake. A level slightly lower than this was observed when membrane filters were counted to determine retention of $^{14}C$ in an experiment which excluded cells (results not shown). Thus, for all practical purposes, the uptake curves illustrated were assumed to represent $^{14}C$-glycerol entering the cell.

Tsay (1971) had originally demonstrated that osmotic shocking of PA-1 produced some loss of ability to transport $^{14}C$-glycerol. However, by altering the shocking technique slightly and correcting for the viability of the assayed suspension by plating, it was possible to show in the present study almost total loss of transport ability by fragile rods (Figure 3b). Procedures used in the present study also resulted in somewhat higher binding ratios, seemingly indicative of greater shocking efficiency.

Although Tsay (1971) suggested that glycerol transport by P. aeruginosa was inducible, transport studies were carried out on only

one set of cells grown on a substrate other than glycerol. $^{14}C$-glycerol uptake by glucose-grown cells was definitely reduced but not to the level expected from free diffusion ($\sim$30 dpm/ml of cell suspension). Therefore, in the present study, a detailed investigation of induction of glycerol transport and binding in wild-type cells was undertaken.

Initially, the induction pattern for glycerol transport was compared with those for glycerol kinase and L-$\alpha$-glycerophosphate dehydrogenase activities (Figure 4 and Table II). The data suggest that one component of the transport system is induced earlier than the rest, since the 30-second value stabilized approximately two hours before the values for the transport plateau and enzyme activities. This supports the hypothesis agreed upon by Winkler and Wilson (1966), Manno and Schachter (1970), Wong and Wilson (1970), and others, which proposes that two transport mechanisms are in operation during active influx of some substrates. The first is independent of cellular metabolism requiring only a binding protein and spontaneous release of substrate. This could be the primary sequence after two hours of induction. The second mechanism requires metabolism for energy production and, in this instance, may account for the plateau accumulation which becomes stable at four hours. Interestingly enough, the activities of the glycerol degradative enzymes which reach their peak at four hours, are progressively lost during the period between four and eight hours. Since, by four hours, most of the substrates have been removed and growth has virtually stopped, it is possible that enzyme degradation, similar to that described by Cowen (1968), has begun. Considering that transport activity from four to eight hours remains constant, it

appears that the specific glycerol degradative enzymes are not directly related to maintenance of the level of glycerol accumulation. Furthermore, it appears that full induction of transport requires four hours.

Upon closer analysis, these data illustrate an additional point of interest. Glycerol uptake by glucose-grown cells (Figure 5d) is greater than that by glucose-grown cells induced up to one hour in a combination glycerol-glucose substrate. This effect is reflected only in the transport curve. The enzyme activities continue to increase from zero-time values of 0.63 and 0.18 nmoles/min/mg for the kinase and dehydrogenase, respectively (Table VI), to 4.95 and 5.56 nmoles/min/mg at one hour into induction (Table II). Repression by glucose does not seem to provide a reasonable explanation since, under this condition, the level of uptake should have remained the same as that in Figure 5d, not decreased (Paigen and Williams, 1970). Instead, catabolite inhibition seems a more feasible possiblity. According to Paigen and Williams (1970), this type of inhibition is either exerted at the level of entry of substrates into the cell (by competition) or on the first enzyme that catalyzes metabolism of the substrate. Glucose was found to be an ineffective inhibitor of glycerol kinase from an _in vitro_ assay (results not shown). Therefore, the inhibition is concluded to be competitive in nature. Since little growth was occurring during the period between zero time and one hour, and there was only slight removal of substrates, it may be assumed that protein synthesis was proceeding at an uninduced rate. Thus, synthesis of additional binding protein was insignificant. Under these circumstances, it is possible that the extremely high levels of glucose and glycerol in the medium could cause saturation of the available glycerol binding sites. If

these substrates were not completely removed during the wash, they could cause retardation of $^{14}$C-glycerol entry. As soon as the substrate concentration was reduced to a low enough level (by two hours into induction) the inhibition was overcome.

Figure 5a-5d suggests not only that full capacity to transport glycerol requires induction by glycerol, but that the levels of glycerol binding and glycerol uptake by uninduced PA-$1_L$ (cells not exposed to glycerol) vary considerably. It is noted that of the four growth substrates used in this experiment, the only one which left the cells unable to bind (binding ratio, $R_S$, has a value less than 1.00) and significantly transport glycerol was succinate. The level of glycerol uptake by these succinate-grown cells was lower than that by any other set of cells assayed (Figures 5 and 6). In addition, Figure 10b indicates that no glycerol binding peaks are present in PPBPS obtained from succinate-grown PA-$1_S$.

Some of the succinate-related results such as inability of shock fluids from succinate-grown cells to bind glycerol, could be explained by these cells being insensitive to osmotic shock. However, this is unlikely in the present situation since the succinate-grown cells also exhibited almost no ability to take up glycerol. A second possibility is that succinate represses a normally constitutive synthesis of components involved with glycerol accumulation. If this were true, all the other substrates tested (Figures 5 and 6) must also produce some degree of repression because none of these substrates yielded cells capable of transporting glycerol at a rate approaching that for glycerol-grown PA-1. Since it is improbable that so many diverse compounds would cause repression of this system, it is concluded that

glycerol binding and subsequent transport are inducible and that succinate is one of the few growth substrates for P. aeruginosa which does not induce either activity.

Table VI indicates that the activities of the glycerol degradative enzymes in glucose-, lactate-, and succinate-grown cells were approximately equivalent, yet glycerol accumulation and glycerol binding activities displayed by these cells differed appreciably (Figure 5a-5d). As the binding ratios increased, so did the initial rates of glycerol uptake. Furthermore, Figures 9a, 9b, and 10a illustrate that there are at least two and possibly three different pH values at which glycerol can be bound, suggesting the presence of several glycerol binding proteins in shock fluids from wild-type cells, these being inducible under different conditions. These binders are thought to be directly related to glycerol transport since an increase in amounts of these components (as determined by the binding ratio) results in an increased initial rate of transport (Figure 5a-5d). Thus, from an analysis of initial transport rates (Figures 5a-5d and 6), it appears that succinate induces no glycerol binders (Figures 5a and 10b), ethylene glycol induces only small amounts, if any (Figure 6), and gluconate, 1,3-propanediol, fructose, histidine, 1,2-propanediol, n-propanol, pyruvate, glyceric acid, lactate, and glucose induce increasingly larger amounts of the glycerol binding components.

After a detailed analysis of shock fluids from wild-type cells containing glycerol binding activity, no correlation between the presence of glycerol kinase or L-α-glycerophosphate dehydrogenase activities and binding activity was found. The following points are important in this connection:

(1) Glycerol kinase activity was generally absent in shock fluids containing glycerol binding activity.

(2) The one shock fluid which did display the kinase activity retained it after freezing and thawing, whereas during this same treatment the binding activity was lost (Table III). In addition, it may be noted that cell-free extracts were consistently stored (being frozen and thawed several times) as long as one year without observing any loss of either enzyme activity; but wild-type shock fluids could never be frozen for any period of time without showing a substantial loss of binding activity.

(3) The various activities (binding, kinase, and dehydrogenase) displayed different sensitivities to inhibitors (Table IV). Glycerol binding was inhibited by sodium azide, sodium arsenate, sodium cyanide, hydrazine, and NEM. Of these, neither sodium azide, sodium arsenate, nor hydrazine, at elevated concentrations, caused inhibition of either enzyme activity. Sodium cyanide inhibited only the kinase and NEM completely inhibited only the dehydrogenase. These two enzyme inhibitions are not particularly disturbing since it is quite possible that cyanide could interfere with the coupled in vitro kinase assay itself and that the L-α-glycerophosphate dehydrogenase is like other such dehydrogenases which are known to contain sulfhydryl groups (Telegdi and Keleti, 1964) and be sensitive to N-alkylmaleimides (Anderson, et al., 1970). Tsay (1971) concluded that the effect of NEM on glycerol binding indicated the presence of sulfhydryl groups in this carrier protein. The inhibition caused by hydrazine is thought to be due to alteration of the pH in the shock fluid, since this compound produces a significant pH change and such changes, even if they are slight, can

greatly reduce the binding capacity (Figures 9, 10 and 11). However, it is doubtful that this interpretation explains the inhibitions by sodium azide, sodium cyanide, or sodium arsenate.

These inhibition patterns as well as that produced by hydrazine could merely be caused by the high reactivity of these compounds. It was for this reason that Anraku (1968b) checked for possible inhibition of galactose and leucine binding by sodium azide. He found these activities to be reduced an insigificant 20 per cent. Pardee (1966), on the other hand, reported the $SO_4^=$ binding activity to be inhibited 50 per cent by potassium cyanide. However, no explanation was given for either effect.

Considering the inhibition patterns produced by sodium azide, sodium cyanide, and 2,4-dinitrophenol in the present investigation, it is tempting to postulate that the shock procedure has removed a membrane particle (part of which functions in glycerol transport) containing components such as those described by Barnes and Kaback (1971) and Kaback and Barnes (1971) and outlined in reactions 31-42 (Chapter I). These researchers suggested that compounds which directly block the transfer of electrons would interfere with the oxidation-reduction cycle along the electron transport chain. Since this cycle is required to induce the conformational change of the binder, allowing subsequent binding and transport, it is possible that no binding would occur in the presence of cyanide or azide. 2,4-Dinitrophenol uncouples oxidative phosphorylation instead of acting directly on the electron transfer mechanism; therefore, this compound would not be expected to inhibit binding.

(4) Addition of ATP or $MgCl_2$ or both (Table V), did not prevent inhibition of glycerol binding activity by sodium azide. Addition of ATP alone to the binding assay reduced glycerol binding activity. However, this is not uncommon since a similar effect was reported by Pardee (1966) who observed a 20 per cent reduction in the $SO_4^=$ binding activity after addition of ATP. Again, the effect could be due to change in pH upon ATP addition as this compound was not neutralized.

(5) Three mutants were characterized as having various lesions affecting either binding and transport or glycerol kinase, but not both. GA-73 displayed binding and transport activities similar to those of PA-$1_S$ (Table VI, Figures 7a, 7c, 11b), but showed no glycerol kinase activity (Table VI). The slight initial activity, which generally fell to zero after three to four minutes of incubation, could have been produced by another kinase since the extracts were not dialyzed and residual amounts of other substrates could have been present in it. AH-8 exhibited only two peaks for binding in its pH binding profile (Figure 11a) both at the same pH, one for glycerol and one for glucose, retained only partial transport ability (Figure 7a-d), but had glycerol kinase activity. Its shock fluid contained significant glycerol and glucose binding activities only when AH-8 was grown in the combination substrate glucose-glycerol or when grown in the presence of either lactate or succinate and induced in the presence of glycerol (Table VI). However, these variable binding capacities may be due to loss of the binding protein(s) as a result of lysis of these cells during the growth cycle (this lytic phenomenon of AH-8 will be discussed in a later section). In addition, a loss of the partial transport ability after shocking (Figure 8a) suggests that some glycerol binder is

present. Interestingly enough, the amount of $^{14}$C-glycerol "uptake" after lysis of fragile rods of AH-8 was greatly increased (Figure 8a). This is thought to result from disruption of the osmotic barrier allowing entrance of substrate and subsequent accumulation of radioactive material via metabolism. This same interpretation is thought to explain the seemingly elevated rate of transport by uninduced AH-8 cells and induced AH-8 cells harvested after the lytic phase. Fragile rods of PA-$1_S$ or PA-$1_L$ react in the same manner after lysis (data not shown) as do those of AH-8 and PA-1-623, even though shocking of the latter mutant has no effect on transport (Figure 8b). This latter observation is in agreement with the rest of the data related to PA-1-623, namely, that this mutant displays no significant peaks in its pH binding profile (Figure 11c), no transport ability under any conditions (Figure 7a-7d), no binding capacity for glycerol in its shock fluids (although it may bind a small amount of glucose when it is grown in the combination substrate glucose-glycerol or in the presence of lactate or succinate and induced in glycerol minimal medium A), yet it has a relatively high constitutive glycerol kinase activity (Table VI).

It was concluded that the tendency of fragile rods to lyse hampered attempts to restore transport ability to osmotically shocked PA-$1_S$ by adding back partially purified binding protein. In an attempt to remove as much binder as possible, the cells were probably lysed since harsh shocking techniques were used (0.05 M Tris-0.5 M sucrose-1.0 mg/ml EDTA) and uptake by the control fragile rods was as great or greater than that of unshocked wild-type cells. Three attempts were made, all before this lytic phenomenon was realized.

Data obtained from the various types of competition experiments (see Tables VII - IX, Figures 12 and 14) were in general agreement considering that complications exist in both the binding and transport assays. In all instances, the control assay results were satisfactory, with "cold" glycerol completely inhibiting binding and transport of $^{14}$C-glycerol and nonradioactive glucose completely inhibiting binding of $^{14}$C-glucose. Although 100 per cent inhibition of binding and transport by a competitor is ideal, many factors can interfere to lower this value. Not only do affinities of single binding proteins vary as to substrate, but it is possible to have more than one protein, each with a distinct $K_m$ for a particular substrate, binding one common substrate. Anraku (1968a) and Rosen (1971) have considered binding inhibition percentages as low as 46 and 19 per cent, respectively, to be indicative of competition. Therefore, each column of values in Tables VII-IX must be analyzed as to all possible interrelationships.

In general, the data in Tables VII and VIII support the proposal made from observation of Figures 9-11 which predicts the existence of multiple glycerol and glucose binders in shock fluid from wild-type P. aeruginosa. There appear to be several distinct binding competition patterns displayed by shock fluids from PA-$1_{S+L}$ grown in various media.

(1) Glycerol binding by glycerol-grown cells shows very little sensitivity to added compounds. Of those which do interfere, n-propanol seems to be the most likely candidate for a true competitor since it did not exhibit effective inhibition in any other assay to which it was added. Furthermore, n-propanol slightly lowered the $^{14}$C-glycerol transport curve after one minute into uptake (results not shown). Dulcitol, adonitol, and glucose are the only other possible

competitors, but all of these interfered to a greater extent with glycerol binding by glucose-grown cells indicating that they are not as specific as n-propanol. The effect produced by histidine hydrochloride may actually be related to pH, since it was not neutralized before addition.

(2) There is no significant correlation between glycerol binding by glycerol- and glucose-grown wild-type cells. Therefore, it is doubtful that these two substrates induce the same glycerol binders.

(3) Glucose binding by both glycerol- and glucose-grown cells shows very similar inhibition patterns indicating that at least several of the same proteins are involved.

(4) Where the activities listed under (3) do not correlate, there is agreement between values for glycerol and glucose binding by glucose-grown cells. However, there is also a great deal of overlap among the latter three sets of values. Again, this suggests involvement of common binders.

(5) Glucose not only completely inhibited the binding of glycerol by glucose-grown cells, but, more specifically, it eliminated the peak for glycerol binding in PPBPS adjusted to pH 5 but not the peak at pH 7 (Table IX). It is this fact, in combination with other data shown in Table IX, that adds support to the hypothesis that peak # 1 at pH 7 (Figure 9b) is glycerol-specific, whereas peak # 1a at pH 5 is nonspecific and, as such, is probably distinct from # 1 even though both appear to be induced only in the presence of glycerol. Additional evidence in this respect is provided by the observation that AH-8 contains peak # 1 but not # 1a.

Electrophoretic analysis of PPBPS from glycerol-grown PA-$1_S$ shows two distinct bands and from three to five faint bands (Clifton F. Savoy, personal communication). There is a good possibility, then, that five binding proteins are involved with the recognition of either glycerol or glucose or both in P. aeruginosa.

Competitors of glycerol transport would be expected to reduce both the binding capacity for glycerol and the rate and total amount of accumulation of this substrate. Since dihydroxyacetone (DHA) was shown to have no detrimental effect on either glycerol or glucose binding by glycerol-grown cells (Tables VII and VIII) but significantly reduced $^{14}$C-glycerol accumulation by these cells (results same as those in Figure 12), it is thought that some phenomenon other than competition is involved upon addition of DHA. For instance, efflux of a $^{14}$C-intermediate from the cells would also lower the apparent rate and amount of $^{14}$C-glycerol uptake. Efflux of $^{14}$C-glycerol itself seems to be an improbable explanation in the present situation because the wild-type cells are metabolizing glycerol. On the other hand, it is known that $CO_2$ is produced during the metabolism of glycerol (Figure 1), this product being radioactive upon catabolism of $^{14}$C-glycerol. Therefore, a comparison was made of $^{14}CO_2$ released from untreated control cells (PA-$1_L$), from cells pretreated with DHA, and from cells pretreated with sodium lactate (Figure 13). It was noted that the effect of nonradioactive DHA on $^{14}$C-glycerol transport by PA-$1_L$ could be explained by increased release of $^{14}CO_2$.

The effect of DL-$\alpha$-glycerophosphate on glycerol binding was not studied since this compound was thought to be contaminated with glycerol. The high concentration added would certainly contain enough

glycerol to alter the binding capacity as measured by $^{14}C$ assay. Also taken into consideration was the fact that strain PA-1, like P. aeruginosa in general, was unable to utilize glycerophosphate for growth. Therefore, even though the addition of DL-$\alpha$-glycerophosphate during measurement of glycerol transport produced a curve similar to those representative of nonmetabolizable competitors (Figure 14b), its true effect cannot be determined until it is purified. Certainly, addition of $\beta$-glycerophosphate in no way altered $^{14}C$-glycerol transport.

Previously-mentioned results have indicated that glucose-grown PA-$1_{S+L}$ can bind and transport some glycerol (Figures 5, 9; Table VI) and, reciprocally, that glycerol-grown wild-type cells can bind glucose. Therefore, it is not surprising that glucose partially inhibits glycerol binding by shock fluids from glycerol-grown cells. However, the expected complementary results are not seen in Figure 12b. Addition of glucose would be expected to cause at least some reduction in transport of $^{14}C$-glycerol. Instead, it stimulated this activity. A closer comparison of Table VII and Figure 12 indicates that, with the exception of glucose, those added compounds which appeared to protect the glycerol binding activity of glycerol-grown cells also stimulated transport of $^{14}C$-glycerol. Transport results after the addition of either of the propanediols or 1,2,4-butanetriol are not shown but demonstrated a similar stimulative effect. (Transport assays to which mannitol, erythritol, glyceric acid, or citrate would have been added were not carried out.)

Attempts to interpret the "lactate effect" according to conventional theories failed. Figure 13 indicates that a reduction in $^{14}CO_2$ output cannot account for the over-all stimulation of glycerol

transport by sodium lactate. Protein synthesis also cannot explain the increased uptake since addition of chloramphenicol (Figure 12) to the cell suspension before measurement of transport had no effect on the stimulation by lactate. Furthermore, Tables VI and X illustrate that the stimulation of transport by addition of either lactate, pyruvate, or glucose to cell suspensions of uninduced wild-type or induced mutants in no way correlated with the enzyme activity levels of these suspensions. Stimulation by each of the compounds was observed in glucose-grown PA-$1_L$ and GA-73 and glucose-grown, glycerol-induced AH-8, but was absent in cells lacking a glycerol or glucose binding protein (succinate-grown PA-$1_L$ and PA-1-623). For these reasons, it is thought that the "lactate effect" may be more directly related to some mechanism involved with binding and/or transport of glycerol than to catabolic enzyme activities as suggested in initial reports by Kaback and Stadtman (1968) on stimulation of glycine uptake.

Wong and Wilson (1970) interpreted a slight stimulation by competitors of $^{14}$C-TMG uptake in uninduced energy-poisoned E. coli as indicating the presence of a small number of membrane carriers. This amount of stimulation increased in proportion to increased numbers of carriers. The theory involved is that compounds sharing the same transport system have the exit pathway blocked during preloading with a competitor. Without an exit process to counterbalance uptake, the cells appear to transport greater quantities of radioactive substrate. Data from Table X show agreement with their results. However, reciprocal exit counterflow (countertransport) experiments, described initially by Rickenberg, et al., (1956) and since used extensively to prove substrate competition (Rosenberg and Wilbrandt, 1957; Halpern, 1967;

Piperno and Oxender, 1968; Wong and Wilson, 1970) indicate that these stimulative compounds are not competitors of $^{14}$C-glycerol transport (Figures 14a-14d), just as they are not competitors of glycerol binding (Tables VII-IX). Figures 14a-14d illustrate that their addition to the transport assay mixture produced no $^{14}$C loss. In fact, stimulation remained prominent in most cases.

Since the earliest investigations involving transport (Cohen and Monod, 1957), the required energy source for this process has been assumed to be ATP. Unrelated carbon sources which stimulated uptake were thought to supply added energy through their own metabolism. Such a theory could explain the "lactate effect" if glycerol transport can be shown to be energy-dependent, a fact which is difficult to prove. Christensen (1962) has stated that the only positive method for proof of active transport is to demonstrate accumulation, against a gradient, of the unchanged substrate inside the cell. In the present investigation of glycerol transport, it is noted that induced wild-type cells accumulate approximately 400 times the extracellular glycerol concentration of 0.636 μM (assuming 80 per cent of the cell weight to be available water). However, this does not assure the involvement of active transport because the cells could be accumulating $^{14}$C-intermediates through metabolism of the $^{14}$C-glycerol. Data from Table XIII suggest the possibility that such intermediates are accumulated. However, neither radioactive peak is identifiable as either glycerol, glycerophosphate, dihydroxyacetone, glyceraldehyde, glyceric acid or dehydroxyacetone phosphate. This fact suggests the conclusion that cells used in the boiling-water extraction experiments (Tables XII and XIII) continued metabolizing after filtration. It is difficult to

postulate the extent of this metabolism since no metabolic inhibitor was added to the wash solution due to the extraneous effects such compounds might have on the cells. However, accumulation of $^{14}C$-products could theoretically have been occurring while the cells on the filter were being washed, while the filters were being transferred to flasks, and even for a while after addition of boiling water to extract the cells. Therefore, the primary purpose for inclusion of Tables XII and XIII is to point out major differences between PA-$1_L$ and GA-73 and not to demonstrate accumulation of glycerol within the cell.

Most researchers use inhibition of transport by inhibitors of oxidative phosphorylation as one effective determinant of active transport. These compounds (generally cyanide, azide, and 2,4-dinitrophenol) are thought to inhibit the cell's energy supply mechanism. Since energy is supposed to convert the carrier to a low affinity form at the inner face of the membrane and cause release of substrate, against the concentration gradient inside the cell, its loss is consequential (Winkler and Wilson, 1966; Piperno and Oxender, 1968). In the absence of energy the affinity of carrier for substrate remains high and an increased rate of exit results in an apparent reduced influx (lower rate of transport). Therefore, the uptake curve resulting from pre-incubation with inhibitors should show rapid equilibration of external and internal concentrations of substrate (facilitated diffusion) because carriers should not be affected. In the present study, earlier experiments demonstrated that both sodium azide and sodium cyanide do affect the glycerol carrier of wild-type P. aeruginosa (Tables VII and VIII). Thus, their inhibition of glycerol transport was not unexpected and cannot be considered necessarily a result of energy loss (Figures

15a, 15b, 15d). In fact, inhibition of binding by sodium cyanide is substantiated by curves in Figures 15b and 15d. Preincubation of cells in 10 mM sodium cyanide reduces the number of carriers, but does not inhibit all of them as the curve is not linear. On the other hand, a 30 mM concentration of this compound inactivates all binders producing a passive diffusion-type uptake curve. Sodium azide, at 30 mM, appeared to be even more inhibitory than sodium cyanide.

The fact that neither oligomycin nor antimycin A inhibit $^{14}$C-glycerol binding (Table IV) or transport (Figures 16a, 16d) is not particularly disturbing. These antibiotics are used most often with cell-free mammalian extracts and have been found to have little effect on bacterial respiratory systems (Smith, 1961). In addition, Barnes and Kaback (1971) now feel that earlier reported antimycin A inhibition of $\beta$-galactoside transport by membrane vesicles of E. coli (Kaback and Milner, 1970) could have been due to induction of passive leakage due to detergent effects of this compound (Marquis, 1965) rather than to inhibition of respiration.

Inhibition of $^{14}$C-glycerol transport by 2,4-dinitrophenol appears to be the most reliable indicator of active transport involvement in the present investigation. This compound does not inhibit the initial enzymes of glycerol catabolism (Table IV), does not inhibit glycerol binding by glycerol-grown wild-type cells (Tables IV, VII, and VIII), yet does inhibit $^{14}$C-glycerol transport. The level of $^{14}$C-glycerol transport remaining after preincubation with 2,4-dinitrophenol is similar to that described by others (Winkler and Wilson, 1966; Kaback and Stadtman, 1968; Kay and Gronlund, 1969; Manno and Schachter, 1970) and is interpreted as indicative of the amount of facilitated diffusion

in these cells.

Theoretically, energy inhibitors added during the transport assay should produce exit counterflow of an actively-accumulated $^{14}C$-substrate. Furthermore, analysis of the effluent (supernatant containing the released material) should yield unaltered substrate in order to prove involvement of active transport. Addition of either sodium cyanide, sodium azide, or 2,4-dinitrophenol during measurement of $^{14}C$-glycerol accumulation produced such a $^{14}C$ loss and, at least as a result of sodium azide addition, the initial effluent (representing a 30 per cent $^{14}C$ loss) contained mostly $^{14}C$-glycerol. A small amount of $^{14}C$-glyceraldehyde or $^{14}C$-dihydroxyacetone phosphate was present in this supernatant, but this could have resulted from metabolic activity between 150 and 180 seconds. Certainly, the peak representing this compound was significantly greater in the 210-second sample. Azide did not seem to affect metabolic activity, for a peak correlating to $^{14}C$-dihydroxyacetone also appeared in the effluent by 240 seconds. Oddly enough, no $^{14}C$-glycerophosphate was detected. Since the transport of glycerol reaches its maximum level (approximately 400 times the extracellular glycerol concentration) by 120 seconds, and the $^{14}C$ loss resulting from azide addition at 135 seconds is significant with the major component being $^{14}C$-glycerol, it seems probable that an energy-dependent mechanism is involved. Perhaps this is the reason that glycerol-grown wild-type cells show a less marked response to inhibitors than do those which are lactate-grown and glycerol-induced. The former may contain lower energy reserves which cause them to take up less glycerol. The $^{14}C$ loss is less significant but is relatively comparable.

It is not likely that any active glycerol transport by P. aeruginosa could be associated with the PEP phosphotransferase system. First of all, according to Phibbs and Eagon (1970), there is no PEP phosphotransferase system in P. aeruginosa. In fact, there are no reports of this mechanism in any members of the genera of Order Pseudomonadales. In the second place, common energy inhibitors such as sodium azide have been shown to stimulate rather than inhibit a PEP system (Roseman, 1969). Finally, although the pleiotropic enzyme $I^-$ mutants (ctr mutants) of E. coli are unable to utilize glycerol along with numerous other carbohydrates, the reason does not appear to be directly related to the enzyme I deficiency. The inability of the $I^-$ mutant to grow on glycerol was shown to be genetically suppressed by: (1) constitutivity of the glycerol enzymes (Berman, et al., 1970); (2) a mutation which abolished the kinetic feedback control of glycerol kinase (Zwaig and Lin, 1966); or (3) a promotor-like mutation that produced increased glycerol kinase activity (Lin, 1970).

The most recent reports of stimulation of active transport by compounds such as lactate and succinate correlate this effect to interaction of the compounds with dehydrogenases coupled to the electron transport chain; this interaction increases the electron flow and, in turn, supplies additional energy for transport (Barnes and Kaback, 1970). Specifically, D-lactic dehydrogenase in E. coli, $\alpha$-glycerophosphate dehydrogenase in Staph. aureus, and both L-$\alpha$-glycerophosphate dehydrogenase and L-lactic dehydrogenase in Bacillus subtilis (Konings, et al., 1971) have been implicated in linkage to transport in membrane preparations of the respective organisms. Recently, Eagon (1971) has suggested the coupling of glucose transport to membrane-bound glucose

dehydrogenase in P. aeruginosa because of stimulation of its uptake by 2-deoxyglucose. Since P. aeruginosa is a strict aerobe and is thought to contain an extensive network of cytochromes (Smith, 1968), it is feasible that several dehydrogenases could be coupled to this transfer system. Certainly, each of the stimulative compounds mentioned in Figure 13 is closely associated to a dehydrogenase. Thus, it is once again tempting to postulate that the energy supply for glycerol transport in P. aeruginosa results from coupling of dehydrogenases to the electron transfer chain and that the shock fluids assayed in the present investigation contain membrane particles large enough to house the cytochromes that make up this electron transfer chain. In this case, lactate, pyruvate, etc., rather than protecting against loss of binding activity, might actually be interacting with coupled dehydrogenases and increasing the electron flow which consequently increases the binding capacity for glycerol by producing a configurational change in the carrier. The discrepancy between stimulation of binding and transport by glucose and succinate may be significant in determining which dehydrogenases are coupled. If glucose dehydrogenase and succinate dehydrogenase (not shown in Figure 1) were directly associated with electron transfer), their respective substrates would be expected to stimulate binding. Since they do not, it is possible that their effect on transport results from their metabolism to intermediates which do interact with coupled dehydrogenases (perhaps to gluconate dehydrogenase in the case of glucose and to pyruvate dehydrogenase in the case of succinate). In this respect, it is noteworthy that stimulation by succinate occurs late (during the plateau) and glucose is not as effective a stimulator as lactate or pyruvate. Perhaps the longer

the shock fluid remains without the presence of compounds which can act as an energy source, the lower the capacity for glycerol binding becomes, until it reaches a minimal constant level. Hence, $Control_1$ and $Control_2$ (Tables VII and VIII) could indicate the maximal and minimal capacities in the absence of additives. Inactivation of transport by energy inhibitors could be variable as there are numerous loci which could be affected. Barnes and Kaback (1971) and Kaback and Barnes (1971) have discussed several such possibilities including: (1) inhibitors which block electron transfer between the site of energy coupling and cytochrome $a_2$ causing efflux; (2) inhibitors which act before the site of energy coupling, slowing the rate of reduction of the energy coupling site but not its rate of oxidation by cytochrome $b_1$, manifesting diminished initial rates of uptake; (3) compounds, such as oxamate for D-lactic dehydrogenase, which specifically inhibit one of the membrane-bound dehydrogenases; and (4) compounds which act directly on the carrier (oxidizing the sulfhydryl group) to inactivate it.

In the present study, the limited inhibitor data correlated with that specified by Barnes and Kaback (1971) and Kaback and Barnes (1971). Sodium azide and sodium cyanide produced results indicating that their sites for inactivation fall between the binding protein coupling sites and cytochrome $a_2$, whereas the results for NEM indicate that the carrier must contain sulfhydryl groups as suggested by Tsay (1971). 2,4-Dinitrophenol gave results similar to those obtained with membrane vesicles of *E. coli* (inhibition of transport). However, Barnes and Kaback (1971) did not elaborate on this effect of 2,4-dinitrophenol since it did not affect D-lactate oxidation.

To date there is increasing evidence that active transport systems in E. coli obtain energy from direct coupling of various dehydrogenases to the electron transfer chain which, in turn, transports the electrons to the incorporated carrier proteins. Data from the present investigation of glycerol transport in P. aeruginosa could be interpreted as supportive of a similar mode of coupling here but are inconclusive. Further study in this area is warranted since this may be a general mechanism for active transport.

The enzyme data for the mutants used in this study are pertinent in helping define control mechanisms and pathways for glycerol catabolism in P. aeruginosa. Both AH-8 and PA-1-623 are L-α-glycerophosphate dehydrogenase negative and constitutive for glycerol kinase. Other studies with PA-1-623 (Cowen, 1968; Mose, 1970) have indicated it to be a deletion mutant since it never reverts and has multiple defects. AH-8 reverts only at a very low rate but has some binding and transport activities so it is possible that its lesion covers a much smaller area. Some portion of the operator region or the regulator gene must be affected in order to produce constitutivity. Since Lin's group (Cozzarelli, et al., 1968; Berman-Kurtz, et al., 1971) has shown the aerobic L-α-glycerophosphate dehydrogenase locus to be adjacent to that for the regulator gene in E. coli, it seems likely that the same linkage may occur in P. aeruginosa because AH-8 and PA-1-623 apparently have a lesion affecting at least these two sites. Assuming that one operon controls glycerol entry and catabolism and that the repressor is nonfunctional, the remaining glycerol activities should be constitutive. This appears to be the case for AH-8. Although transport is only partial, the level remains essentially constant whether the cells

are induced or not (Figure 7). Since PA-1-623 displays the expected constitutive glycerol kinase activity but exhibits no binding or transport activities, the proposal of two distinct loci for kinase and transport is further substantiated.

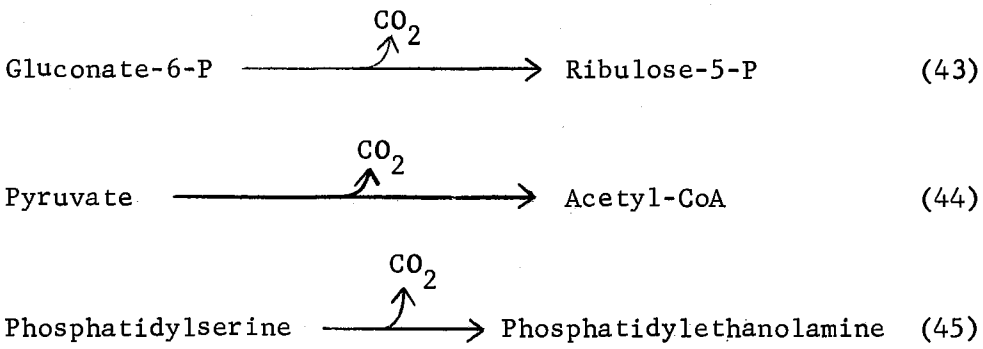
Of the two dehydrogenase negative strains, AH-8 is complicated by the fact that it requires glycerol in order to avoid lysis during logarithmetic growth. Lysis of this mutant almost always occurs during the stationary phase but this is thought to be due to earlier exhaustion of the glycerol supply. This lytic effect may either be directly related to the absence of a biosynthetic glycerophosphate dehydrogenase, assuming this enzyme to be present in P. aeruginosa as it is in E. coli (Kito and Pizer, 1968, 1969), or to the absence of some other enzyme which would function at the branchpoint to the biosynthetic pathway for phospholipid production from substrates other than glycerol. Because phospholipids are thought to be a major component of cell membranes (Day and Levy, 1969), it is hypothesized that AH-8 can only synthesize a defective structure unless glycerol is provided in the medium (constitutive glycerol transport and kinase activities allow synthesis of glycerophosphate and probably phospholipids from glycerol under any condition). In this respect, PA-1-623 must differ enzymatically. Since it cannot transport glycerol, this mutant cannot synthesize phospholipid from glycerol. Since it also lacks the catabolic L-$\alpha$-glycerophosphate dehydrogenase, the activity of this enzyme in the direction of glycerophosphate synthesis is not available for synthesis of phospholipid. Therefore, the combination of loss of ability to transport glycerol and ability to convert DHAP to L-$\alpha$-glycerophosphate should be lethal unless a third pathway for synthesis of phospholipid

is possible or unless there are two enzymes functioning (one catabolic, L-$\alpha$-glycerophosphate dehydrogenase, and one biosynthetic) in the region of reaction 6 (or possibly 4) in Figure 1.

The behavior of GA-73 is unexplainable from present available information although it raises some interesting questions about glycerol catabolism in P. aeruginosa. The fact that this mutant transports glycerol in the absence of glycerol kinase indicates that the latter enzyme is uninvolved with the glycerol transport mechanism. However, additional enzyme data coupled with $^{14}CO_2$ accumulation results (Table XIV) and chromatography information (Tables XII, XIII) suggest a major metabolic difference between GA-73 and PA-1 or AH-8, implying the presence of an unreported pathway for glycerol catabolism in P. aeruginosa.

Assuming the presence of a single glycerol catabolic pathway (Figure 1, reactions 1, 4, 6, 9), mutant GA-73 should be unable to metabolize glycerol since it has no glycerol kinase activity. Yet cell suspensions of this mutant produce $^{14}CO_2$ from $^{14}C$-glycerol and unidentifiable $^{14}C$-intermediates are found in boiling-water extracts of GA-73 exposed to radioactive glycerol (Tables XII and XIII). Interestingly, the same two $^{14}C$-intermediates are found in GA-73 and PA-$1_L$ extracts. However, there is an opposite effect of time on distribution of the two peaks. Since peak # 1 remains at the origin and appears to be charged (the compound it represents seems to bind to DEAE cellulose paper) it is thought to represent phospholipid. Peak # 2 could be glyceraldehyde-3-phosphate. Pretreatment of cell suspensions of GA-73 with DHA prior to measurement of $^{14}CO_2$ accumulation also causes an effect exactly opposite (reduced production of $^{14}CO_2$) to that displayed by PA-$1_L$ under

the same conditions (increased production of $^{14}CO_2$). Furthermore, $^{14}CO_2$ production is immediate (Figure 13) especially after pretreatment of PA-$1_L$ cells with DHA. Unfortunately, possible metabolic decarboxylation reactions assumed or known to occur in carbohydrate metabolism in P. aeruginosa (shown in reactions 43-45) are distant in relation to glycerol.

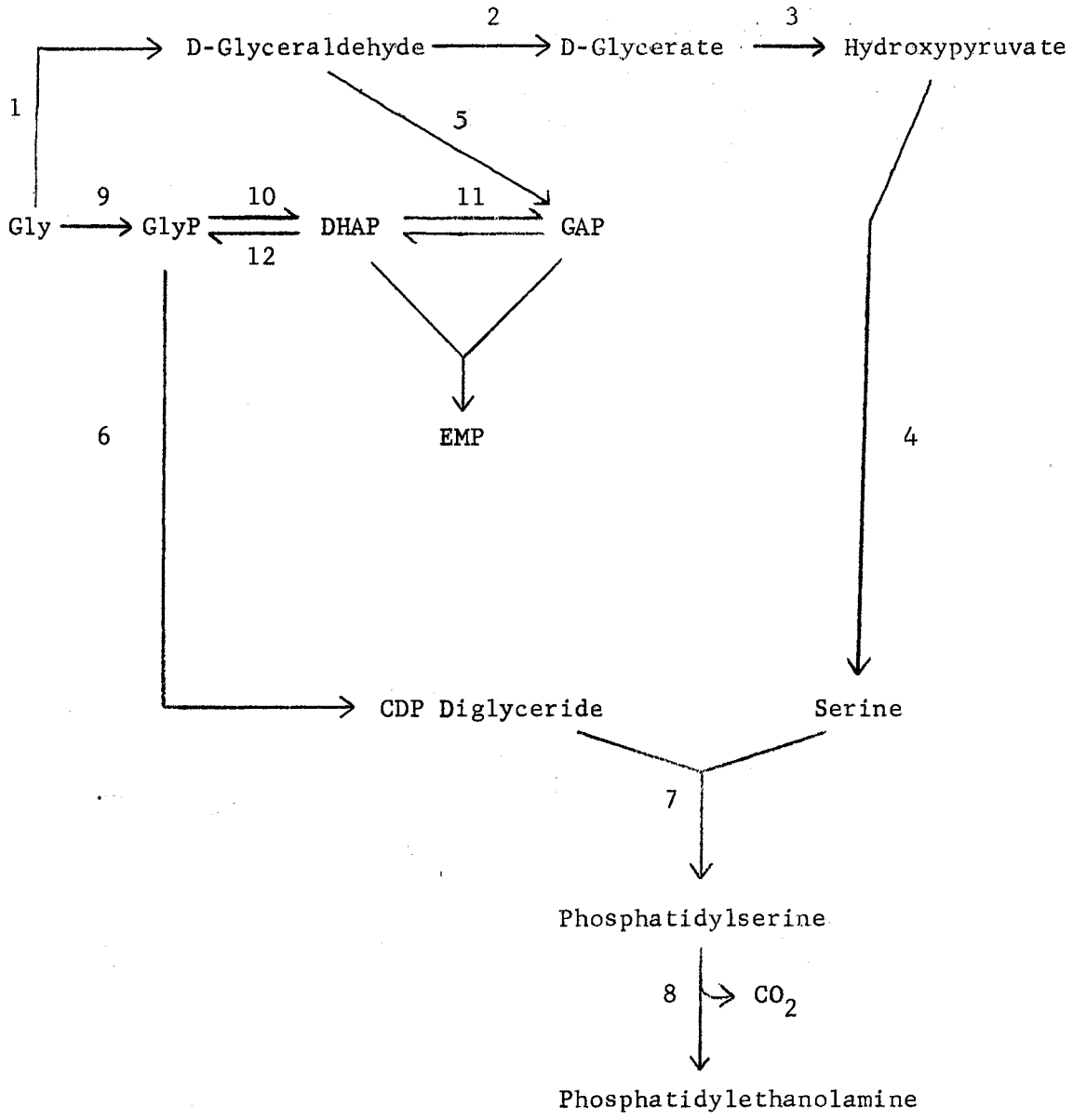



Since GA-73 is unable to grow on glycerol it is improbable that reactions 43 or 44 could account for the $^{14}CO_2$ produced from $^{14}C$-glycerol. These would indicate the presence of extensive metabolic conversions, beginning with glycerol, and would almost certainly assure growth on this substrate. (GA-73 can grow on glucose, lactate, or succinate, each of which must incorporate these reactions.) Recently, Bell, et al. (1971) reported the presence of reaction 45 in E. coli and S. typhimurium. Formation of serine from glycerol is possible according to the metabolic map published by C. F. Boehringer (1965), with the series of reactions (Figure 17) initiated by a $NADP^+$-requiring glycerol dehydrogenase. Cell-free extracts of PA-$1_L$ and GA-73 were spectrophotometrically and radioactively (by radiochromatography) assayed for such an enzyme according to the method of Goldman (1963). Glycerol dehydrogenase activity was found even though conditions were not optimized ($\Delta$O.D./min = 0.022 as compared to $\Delta$O.D./min = 0.060 for

Figure 17. Synthesis of Phosphatidylethanolamine From Glycerol

Abbreviations are as follows:
Gly, Glycerol
GlyP, Glycerophosphate
DHAP, Dihydroxyacetone phosphate
GAP, Glyceraldehyde-3-phosphate
EMP, Emden-Meyerhoff-Parnas pathway

1. Glycerol dehydrogenase
2. D-Glyceraldehyde dehydrogenase
3. Glycerate dehydrogenase
4. Transaminase
5. D-Glyceraldehyde kinase
6. Series of reactions leading to CDP Diglyceride
7. Serine CMP: phosphatidyltransferase
8. Phosphatidylserine decarboxylase
9. Glycerol kinase
10. L-$\alpha$-glycerophosphate dehydrogenase
11. Triose phosphate isomerase
12. Biosynthetic glycerophosphate dehydrogenase


D-Glyceraldehyde
D-Glycerate
Hydroxypyruvate
Gly
GlyP
DHAP
GAP
EMP
CDP Diglyceride
Serine
Phosphatidylserine
Phosphatidylethanolamine
$CO_2$
1
2
3
4
5
6
7
8
9
10
11
12

glycerol kinase and $\Delta$O.D./min = 0.165 for L-$\alpha$-glycerophosphate dehydrogenase in PA-$1_L$). Radioactive products formed after 45 min incubation of the assay mixture at room temperature chromatographed in two broad peaks. The $R_G$ of the first peak (0.83) corresponds to that for glyceraldehyde or dihydroxyacetone phosphate ($R_G$ = 0.85), whereas the second peak ($R_G$ = 0.54) could represent glyceric acid ($R_G$ = 0.49). The extracts were not dialyzed so a series of reactions could have occurred. It is noteworthy that Dawson et al. (1969) state that D-glyceraldehyde readily isomerizes to DHA in alkali. This phenomenon may have occurred upon addition of sodium azide to cells accumulating $^{14}$C-glycerol. If so, it would account for the appearance of $^{14}$C-DHA in the supernatant at 105 seconds after azide addition and substantiate the presence of reaction 1 (Figure 17). Since the same level of glycerol dehydrogenase activity was detected in PA-$1_L$ and AH-8 it is doubtful that this enzyme is directly involved with glycerol transport although it may be coupled to electron transport.

Without further investigation of glycerol catabolism in P. aeruginosa it is impossible to fully interpret the data obtained in this study. Nevertheless, there are indications that a pathway yet unreported in P. aeruginosa is present. In total, it could be represented by reactions 1 through 8 in Figure 17. Only reactions 6, 7, 8, 9, 10, 11, 12, and 1 are known to exist in bacteria. Reactions 9, 10, 11, and now 1, have been assayed in P. aeruginosa.

Assuming phosphatidylethanolamine biosynthesis to occur in P. aeruginosa according to the pathway represented by Figure 17, it is thought that GA-73 contains enzymes 1, 2, 3, 4, 5, 6, 7, 8, 11, 12 so that it can produce phosphatidylethanolamine and $CO_2$ from glycerol but

cannot grow on this substrate. On the other hand, PA-$1_L$ contains all the enzymes. The involvement of DHA as reflected by the $^{14}CO_2$ accumulation experiments is not fully understood. However, it must act in a control capacity, more than likely directing its effect on the segment made up of reactions 9, 10, 11, and/or possible 5. This could route all glycerol metabolism in PA-1 through phosphatidylethanolamine biosynthesis and $CO_2$ production upon addition of DHA. The lesion in GA-73 seems to have affected control in general because L-$\alpha$-glycerophosphate dehydrogenase activity is present when this mutant is grown in glucose but not when it is grown in the presence of lactate or succinate. Therefore, the DHA effect, if it is involved with control, may be masked in GA-73.

Inasmuch as glycerol dehydrogenase activity has been detected in P. aeruginosa, it is evident that investigation of the pathways of glycerol catabolism in this organism should be continued. Certainly, more glycerol catabolic enzymes exist than have previously been reported in this organism.

# CHAPTER V

## SUMMARY AND CONCLUSIONS

The binding and transport of glycerol by P. aeruginosa has been investigated and has been found to be facilitated by inducible proteins (binding proteins) which are distinct from the enzymes involved with glycerol catabolism (glycerol kinase and L-α-glycerophosphate dehydrogenase). This was concluded from the following observations.

(1) Only growth in the presence of glycerol produced wild-type cells with full glycerol transport ability. Growth of PA-1 in the presence of glucose, lactate, or any of several other substrates induced partial transport ability, while growth of PA-1 in the presence of succinate produced cells lacking glycerol binders and unable to transport this substrate.

(2) Osmotic shocking of induced wild-type cells reduced the ability of the cells to transport glycerol by 97 per cent. The shock fluid from these cells was shown to bind glycerol and glucose, whereas the shock pellet retained glycerol kinase and L-α-glycerophosphate dehydrogenase activities.

(3) Two of the three mutants isolated during the course of this study exhibited significant variations in binding and enzyme profiles. One (GA-73), was shown to have four of the five binders detected in the present study and to have full transport ability, but no glycerol kinase activity. The second (PA-1-623), lacked all glycerol binders

and was unable to transport glycerol, but displayed a high glycerol kinase activity.

(4) Various inhibitory compounds produced different effects on the enzyme activities and the glycerol binding activity. For example, sodium azide completely inhibited the glycerol binding and transport activities but had no effect on either enzyme activity. Sodium arsenate also inhibited glycerol binding but had no effect on either enzyme activity. NEM inhibited both binding and transport but only slightly lowered glycerol kinase activity at a 10-fold higher concentration. Sodium cyanide inhibited binding and transport of glycerol, yet it was a requirement in the L-α-glycerophosphate dehydrogenase assay. It inhibited kinase only at a 10-fold higher concentration. Finally, hydrazine was required as a component of the glycerol kinase assay and stimulated the dehydrogenase reaction when added to the assay mixture, yet it completely eliminated the glycerol binding activity.

(5) Neither ATP nor $MgCl_2$ nor a combination of the two exhibited a protective effect on the glycerol binder in the presence of sodium azide.

Studies on partially purified carrier proteins coupled with results from binding competition experiments revealed an interrelationship between glycerol and glucose binding. Five binding proteins were tentatively identified as inducible by glycerol or glucose. Furthermore, glycerol was shown to completely inhibit glucose binding in shock fluid from glycerol-grown cells, and, reciprocally, glucose partially inhibited glycerol binding by this shock fluid. The interaction of the two substrates was also evident in shock fluid from glucose-grown cells. The major protein involved specifically with glycerol binding

had a pH optimum for binding at pH 7 and was inducible only by glycerol. A second carrier inducible only by glycerol and binding optimally at pH 5, was nonspecific. It appeared to bind such unrelated compounds as glucose, lactate, and pyruvate. Two carrier proteins which appeared at low levels even in a partially purified binding protein preparation from succinate-grown PA-$1_S$, bound glucose at optimum pH's of 7 and 5 but were determined to be distinct from the previous ones since these glucose binders were constitutive. Their specificity was not determined. Finally, a protein which bound either glucose or glycerol optimally at pH 9 was demonstrated to be inducible by glucose.

Results of binding and transport competition experiments were not in agreement. Only n-propanol could be shown to affect both activities. However, the affinity of the glycerol-specific carrier for n-propanol was low since this compound at a high concentration relative to that of $^{14}$C-glycerol, only partially inhibited glycerol binding and transport. A number of the competitors of glycerol binding by glucose-grown cells produced a stimulatory effect on glycerol transport rather than a slightly inhibitory one as expected. This fact, coupled with results of counterflow experiments, suggested two properties of glycerol transport.

(1) The transport of glycerol by wild-type P. aeruginosa is specific, perhaps involving only the glycerol-specific binder.

(2) Transport of glycerol by P. aeruginosa is an active process which is stimulated by metabolizable compounds.

With respect to the latter possibility, it was noted that common inhibitors of electron transfer (sodium azide and sodium cyanide) inhibited both binding and transport of glycerol, whereas an uncoupler

of oxidative phosphorylation (2,4-dinitrophenol) inhibited only the latter activity. Furthermore, addition of these compounds to wild-type cells preloaded with labeled glycerol caused an efflux of $^{14}C$ (consisting mainly of glycerol). The initially released material accounted for approximately 30 per cent of the glycerol taken up by the cells and represented an intracellular concentration of glycerol 120 times greater than the external concentration of this substrate. The internal $^{14}C$ concentration at 150 seconds into transport was calculated to be 400 times greater than the external concentration but some of this may be present as metabolic products of glycerol. It was thus concluded that the transport of glycerol by P. aeruginosa is an active process possibly receiving energy via direct coupling of binders to the electron transfer chain as suggested by other investigators.

Comparison of glycerol metabolism by a glycerol kinase negative mutant (GA-73) and wild-type cells indicated the presence of a second unreported pathway for glycerol catabolism in P. aeruginosa. Additional studies in this area confirmed the presence of a glycerol dehydrogenase which appears to convert glycerol to D-glyceraldehyde.

## BIBLIOGRAPHY


Albers, R. W. 1967. Biochemical aspects of active transport. Ann. Rev. Biochem. 36: 727-756.

Anderson, B. M., S. J. Kim, and C. N. Wang. 1970. Inactivation of rabbit muscle L-$\alpha$-glycerophosphate dehydrogenase by N-alkylmaleimides. Arch. Biochem. Biophys. 138: 66-72.

Anderson, B., W. Kundig, R. Simoni, and S. Roseman. 1968. Further studies of carbohydrate permeases. Fed. Proc. 27: 643.

Anraku, Y. 1967. The reduction and restoration of galactose transport in osmotically shocked cells of Escherichia coli. J. Biol. Chem. 242: 793-800.

Anraku, Y. 1968a. Transport of sugars and amino acids in bacteria. I. Purification and specificity of galactose- and leucine-binding proteins. J. Biol. Chem. 243: 3116-3122.

Anraku, Y. 1968b. Transport of sugars and amino acids in bacteria. II. Properties of galactose- and leucine-binding proteins. J. Biol. Chem. 243: 3123-3127.

Anraku, Y. 1968c. Transport of sugars and amino acids in bacteria. III. Studies on the restoration of active transport. J. Biol. Chem. 243: 3128-3135.

Anraku, Y., and L. A. Heppel. 1967a. Changes induced in E. coli by osmotic shock. Fed. Proc. 26: 393.

Anraku, Y., and L. A. Heppel. 1967b. On the nature of the changes induced in Escherichia coli by osmotic shock. J. Biol. Chem. 242: 2561-2569.

Barnes, E. M., Jr., and H. R. Kaback. 1970. $\beta$-Galactoside transport in bacterial membrane preparations: energy coupling via membrane-bound D-lactic dehydrogenase. Proc. Nat. Acad. Sci. U. S. A. 66: 1190-1198.

Barnes, E. M., Jr., and H. R. Kaback. 1971. Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and $\beta$-galactoside transport in Escherichia coli membrane vesicles. J. Biol. Chem. 246: 5518-5522.

Barrett, J. T., A. D. Larson, and R. E. Kallio. 1953. The nature of the adaptive lag of Pseudomonas fluorescens toward citrate. J. Bacteriol. 65: 187-192.

Beckwith, J. R., and D. Zipser. 1970. The lactose operon. Cold Spring Harbor Laboratory, New York.

Bell, R. M., R. D. Mavis, M. J. Osborn, and P. R. Vagelos. 1971. Enzymes of phospholipid metabolism: localization in the cytoplasmic and outer membrane of the cell envelope of Escherichia coli and Salmonella typhimurium. Biochim. Biophys. Acta 249: 628-635.

Berman, M., and E. C. C. Lin. 1971. Glycerol specific revertants of a phosphoenolypyruvate phosphotransferase mutant: suppression by the desensitization of glycerol kinase to feedback inhibition. J. Bacteriol. 105: 113-120.

Berman, M., N. Zwaig, and E. C. C. Lin. 1970. Suppression of a pleiotropic mutant affecting glycerol dissimilation. Biochem. Biophys. Res. Commun. 38: 272-278.

Berman-Kurtz, M., E. C. C. Lin, and D. P. Richey. 1971. Promoter-like mutant with increased expression of the glycerol kinase operon of Escherichia coli. J. Bacteriol. 106: 724-731.

Bhattacharyya, P. 1970. Active transport of manganese in isolated membranes of Escherichia coli. J. Bacteriol. 104: 1307-1311.

Boos, W. 1969. The galactose binding protein and its relationship to the $\alpha$-methylgalactoside permease from Escherichia coli. Eur. J. Biochem. 10: 66-73.

Breed, R. S , E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology. (7th ed). The Williams & Wilkins Company, Baltimore.

Carter, J. R., C. F. Fox, and E. P. Kennedy. 1968. Interaction of sugars with the membrane protein component of the lactose transport system of Escherichia coli. Proc. Nat. Acad. Sci. U. S. A. 60: 725-732.

Chabner, B., and D. Livingston. 1970. A simple enzymatic assay for pyridoxal phosphate. Anal. Biochem. 34: 413-423.

Christensen, H. N. 1962. Biological transport. W. A. Benjamin, Inc. New York.

Cohen, G. N., and J. Monod. 1957. Bacterial permeases. Bacteriol. Rev. 21: 169-194.

Cohen, G. N , and H. V. Rickenberg. 1955a. Etude directe de la fixation d'un inducteur de la $\beta$-galactosidase par les cellules d'Escherichia coli. Compt. Rend. 240: 466-468.

Cohen, G. N., and H. V. Rickenberg. 1955b. Existence d'accepteurs spécifiques pour les aminoacides chez Escherichia coli. Compt. Rend. 240: 2086-2088.

Cohen, G. N., and H. V. Rickenberg. 1956. Concentration spécifique réversible des aminoacides chez Escherichia coli. Ann. Inst. Pasteur 91: 693-720.

Cowen, C. M. 1968. Catabolic pathways and metabolic controls in Pseudomonas aeruginosa. M. S. Thesis, Oklahoma State University, Stillwater.

Cox, S. T., Jr., and R. G. Eagon. 1968. Action of ethylenediaminetetraacetic acid, tris(hydroxymethyl)aminomethane, and lysozyme on cell walls of Pseudomonas aeruginosa. Can. J. Microbiol. 14: 913-922.

Cozzarelli, N. R., W. B. Freedberg, and E. C. C. Lin. 1968. Genetic control of the L-$\alpha$-glycerophosphate system in Escherichia coli. J. Mol. Biol. 31: 371-387.

Davis, E. J., and D. M. Gibson. 1967. Stimulation of phosphoenolpyruvate formation by oleic acid and other uncouplers of oxidative phosphorylation in rabbit liver mitochondria. Biochem. Biophys. Res. Commun. 29: 815-821.

Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones. 1959. Data for biochemical research. Oxford University Press, London.

Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones. 1969. Data for biochemical research. (2nd ed). Oxford University Press, London.

Day, C. E., and R. S. Levy. 1969. A generalized functional role for phospholipids. J. Theoret. Biol. 22: 541-542.

Deere, C. J., A. D. Dulaney, and I. D. Michelson. 1939. The lactose activity of Escherichia coli-mutabile. J. Bacteriol. 37: 355-363.

Doudoroff, M. 1951. The problem of the "direct utilization" of disaccharides by certain microorganisms. p. 42-48. In W. D. McElroy and B. Glass (ed), Symposium on phosphorus metabolism, vol. 1. Johns Hopkins University Press, Baltimore, Maryland.

Doudoroff, M., W. Z. Hassid, E. W. Putnam, A. L. Potter, and J. Lederberg. 1949. Direct utilization of maltose by Escherichia coli. J. Biol. Chem. 179: 921-934.

Doudoroff, M., N. J. Palleroni, J. MacGee, and M. Ohara. 1956. Metabolism of carbohydrates by Pseudomonas saccharophila. I. Oxidation of fructose by intact cells and crude cell-free extracts. J. Bacteriol. 71: 196-201.

Dreyfuss, J., and A. B. Pardee. 1965. Evidence for a sulfate-binding site external to the cell membrane of Salmonella typhimurium. Biochim. Biophys. Acta 104: 308-310.

Eagon, R. G. 1971. 2-Deoxyglucose transportation via passive diffusion and its oxidation, not phosphorylation, to 2-deoxygluconic acid by Pseudomonas aeruginosa. Can. J. Biochem. 49: 606-613.

Egan, J. B., and M. L. Morse. 1965a. Carbohydrate transport in Staphylococcus aureus. I. Genetic and biochemical analysis of a pleiotropic transport mutant. Biochim. Biophys. Acta 97: 310-319.

Egan, J. B., and M. L. Morse. 1965b. Carbohydrate transport in Staphylococcus aureus. II. Characterization of the defect of a pleiotropic transport mutant. Biochim. Biophys. Acta 109: 172-183.

Egan, J. B., and M. L. Morse. 1966. Carbohydrate transport in Staphylococcus aureus. III. Studies of the transport process. Biochim. Biophys. Acta 112: 63-73.

Englesberg, E., J. A. Watson, and P. A. Hoffee. 1961. The glucose effect and the relationship between glucose permease, acid phosphatase, and glucose resistance. Cold Spring Harbor Symp. Quant. Biol. 26: 261-276.

Fisher, A. 1903. Vorlesungen über Bakterien. (2nd ed). Fisher, Jena.

Fox, C. F., J. R. Carter, and E. P. Kennedy. 1967. Genetic control of the membrane protein component of the lactose transport system of Escherichia coli. Proc. Nat. Acad. Sci. U. S. A. 57: 698-705.

Fox, C. F., and E. P. Kennedy. 1965. Specific labeling and partial purification of the M protein, a component of the β-galactoside transport system of Escherichia coli. Proc. Nat. Acad. Sci. U. S. A. 54: 891-899.

Furlong, C. E., and J. H. Weiner. 1970. Purification of a leucine-specific binding protein from Escherichia coli. Biochem. Biophys. Res. Commun. 38: 1076-1083.

Gaby, W. L. 1946. A study of the dissociative behavior of Pseudomonas aeruginosa. J. Bacteriol. 51: 217-234.

Gale, E. F. 1947. The passage of certain amino acids across the cell wall and their concentration in the internal environment of Streptococcus faecalis. J. Gen. Microbiol. 1: 53-76.

Gale, E. F. 1954. The accumulation of amino acids within staphylococcal cells. p. 242-253. In Active transport and secretion, Symposia of the Society for Experimental Biology, vol. VIII. Academic Press, Inc., New York.

Gale, E. F., and J. P. Folkes. 1967. The effect of lipids on the accumulation of certain amino acids by Staphylococcus aureus. Biochim. Biophys. Acta 144: 461-466.

Gale, E. F., and J. M. Llewellin. 1970. Release of lipids from, and their effect on aspartate transport in osmotically shocked Staphylococcus aureus. Biochim. Biophys. Acta 222: 549-551.

Ganesan, A. K., and B. Rotman. 1966. Transport systems for galactose and galactosides in Escherichia coli. I. Genetic determination and regulation of the methyl-galactoside permease. J. Mol. Biol. 16: 42-50.

Gilvarg, C., and B. D. Davis. 1954. Significance of the tricarboxylic acid cycle in Escherichia coli. Fed. Proc. 13: 217.

Goldman, D. 1963. Enzyme systems in the mycobacteria. XV. Initial steps in the metabolism of glycerol. J. Bacteriol. 86: 30-37.

Gordon, H. T., W. Thornburg, and L. N. Werum. 1956. Rapid paper chromatography of carbohydrates and related compounds. Anal. Chem. 28: 849-855.

Gregory, J. D., and S. W. Sajdera. 1970. Interference in the Lowry method for protein determination. Science 169: 97-98.

Hadley, P. 1927. Microbic dissociation. J. Infect. Diseases 40: 1-312.

Halpern, Y. 1967. Further studies of glutamate transport in Escherichia coli. Some features of the exit process. Biochim. Biophys. Acta 148: 718-724.

Harold, F. M., and J. R. Baarda. 1968. Inhibition of membrane transport in Streptococcus faecalis by uncouplers of oxidative phosphorylation and its relationship to proton conduction. J. Bacteriol. 96: 2025-2034.

Hayashi, S., J. P. Koch, and E. C. C. Lin. 1964. Active transport of L-$\alpha$-glycerophosphate in Escherichia coli. J. Biol. Chem. 239: 3098-3105.

Hayashi, S., and E. C. C. Lin. 1965. Capture of glycerol by cells of Escherichia coli. Biochim. Biophys. Acta 94: 479-487.

Heath, H. E., III. 1971. Glucose metabolism and catabolite repression in Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

Hengstenberg, W., J. B. Egan, and M. L. Morse. 1967. Carbohydrate transport in Staphylococcus aureus. V. The accumulation of phosphorylated carbohydrate derivatives, and evidence for a new enzyme splitting lactose phosphate. Proc. Nat. Acad. Sci. U. S. A. 58: 274-279.

Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science 156: 1451-1455.

Ishaque, M., A. Donawa, and M. I. H. Aleem. 1971. Electron transport and coupled energy generation in Pseudomonas saccharophila. Can. J. Microbiol. 49: 1175-1182.

Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

Jacob, F., D. Perrin, C. Sanchez, and J. Monod. 1960. L'operon: groupe de gènes à expression coordonnée par un opérateur. Compt. Rend. 250: 1727-1729.

Jacobs, M. H., H. N Glassman, and A. K. Parpart. 1938. Osmotic properties of the erythrocyte. J. Cellular Comp. Physiol. 11: 479-494.

Jacobs, M. H., H. N. Glassman, and A. K. Parpart. 1950. Hemolysis and zoological relationship. Comparative studies with four penetrating non-electrolytes. J. Expt. Zool. 113: 277-300.

Jacobs, N. J., and P. J. VanDemark. 1960. Comparison of the mechanism of glycerol oxidation in aerobically and anaerobically grown Streptococcus faecalis. J. Bacteriol. 79: 532-538.

Kaback, H. R. 1970. Transport. Ann. Rev. Biochem. 39: 561-598.

Kaback, H. R., and E. M. Barnes, Jr. 1971. Mechanisms of active transport in isolated membrane vesicles. II. The mechanism of energy coupling between D-lactic dehydrogenase and β-galactoside transport in membrane preparations from Escherichia coli. J. Biol. Chem. 246: 5523-5531.

Kaback, H. R., and L. S. Milner. 1970. Relationship of a membrane-bound D-(-)-lactic dehydrogenase to amino acid transport in isolated bacterial membrane preparations. Proc. Nat. Acad. Sci. U. S. A. 66: 1008-1015.

Kaback, H. R., and E. R. Stadtman. 1968. Glycine uptake in Escherichia coli. II. Glycine uptake, exchange, and metabolism by an isolated membrane preparation. J. Biol. Chem. 243: 1390-1400.

Kay, W. W., and A. F. Gronlund. 1969. Proline transport by Pseudomonas aeruginosa. Biochim. Biophys. Acta 193: 444-455.

Kennedy, E. P., and G. A. Scarborough. 1967. Mechanism of hydrolysis of o-nitrophenyl-β-galactoside in Staphylococcus aureus and its significance for theories of sugar transport. Proc. Nat. Acad. Aci. U. S. A. 58: 225-228.

Kepes, A. 1960. Etudes cinétiques sur la galactoside perméase d'Escherichia coli. Biochim. Biophys. Acta 40: 70-84.

Kepes, A. 1971. The β-galactoside permease of Escherichia coli. J. Membrane Biol. 4: 87-112.

Kepes, A., and J. Monod. 1957. Etude du fonctionement de la galactoside-permease d'Escherichia coli. Compt. Rend. 244: 809-811.

Kito, M., and L. I. Pizer. 1968. The biosynthetic L-α-glycerophosphate dehydrogenase of E. coli. Biochem. Biophys. Res. Commun. 32: 408-412.

Kito, M., and L. I. Pizer. 1969. Phosphatidic acid synthesis in Escherichia coli. J. Bacteriol. 97: 1321-1327.

Koch, A. L. 1964. The role of permease in transport. Biochim. Biophys. Acta 79: 177-200.

Koch, A. L. 1967. Kinetics of permease catalyzed transport. J. Theoret. Biol. 14: 103-130.

Koch, A. L. 1971. Energy expenditure is obligatory for the downhill transport of galactosides. J. Mol. Biol. 59: 447-459.

Koch, A. L., and R. Coffman. 1970. Diffusion, permeation, or enzyme limitation: a probe for the kinetics of enzyme induction. Biotech. and Bioeng. 12: 651-677.

Kogut, M., and E. P. Podoski. 1953. Oxidative pathways in a fluorescent Pseudomonas. Biochem. J. 55: 800-811.

Komolrit, K. 1965. Biochemical response of activated sludge processes to organic shock loads. Ph.D. Thesis, Oklahoma State University, Stillwater.

Konings, W. N., E. M. Barnes, Jr., and H. R. Kaback. 1971. Mechanisms of active transport in isolated membrane vesicles. I. The coupling of reduced phenazine methosulfate to the concentrative uptake of β-galactosides and amino acids. J. Biol. Chem. 246: 5857-5861.

Kramer, H. 1935. Neue Untersuchungen über die antagonistiche wirkung des Bacillus pyocyaneus. Z. Immunitäts. 84: 505-534.

Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Proc. Nat. Acad. Sci. U. S. A. 52: 1067-1074.

Kundig, W., F. D. Kundig, B. E. Anderson, and S. Roseman. 1965. D-Galactose-6-phosphate synthesis by a phosphotransferase system. Fed. Proc. 24: 658.

Kundig, W., F. D. Kundig, B. E. Anderson, and S. Roseman. 1966. Restoration of active transport of glycosides in Escherichia coli by a component of a phosphotransferase system. J. Biol. Chem. 241: 3243-3246.

Kundig, W., and S. Roseman. 1966. A phosphoenolpyruvate-hexose phosphotransferase system from Escherichia coli. p. 396-403. In W. I. Wood (ed), Methods in enzymology, vol. 9. Academic Press Inc., New York.

Laue, P., and R. E. MacDonald. 1968a. Identification of thiomethyl-β-D-galactoside 6-phosphate accumulated by Staphylococcus aureus. J. Biol. Chem. 243: 680-682.

Laue, P., and R. E MacDonald. 1968b. Studies on the relation of thiomethyl-β-D-galactoside accumulation to thiomethyl-β-D-galactoside phosphorylation in Staphylococcus aureus. Biochim. Biophys. Acta 165: 410-418.

Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63: 399-405.

LeFevre, P. G. 1948. Evidence of active transfer of certain non-electrolytes across the human red cell membrane. J. Gen. Physiol. 31: 505-527.

Leive, L. 1965a. RNA degradation and the assembly of ribosomes in actinomycin-treated Escherichia coli. J. Mol. Biol. 13: 862-875.

Leive, L. 1965b. A non-specific increase in permeability in Escherichia coli produced by EDTA. Proc. Nat. Acad. Sci. U. S. A. 53: 745-750.

Leive, L. 1965c. Actinomycin sensitivity and Escherichia coli. Biochem. Biophys. Res. Commun. 18: 13-17.

Leive, L. 1965d. Release of lipopolysaccharide by EDTA treatment of Escherichia coli. Biochem. Biophys. Res. Commun. 21: 290-296.

Levinthal, M., and R. D. Simoni. 1969. Genetic analysis of carbohydrate transport-deficient mutants of Salmonella typhimurium. J. Bacteriol. 97: 250-255.

Lin, E. C. C. 1970. The genetics of bacterial transport systems. Ann. Rev. Genetics 4: 225-262.

Lin, E. C. C., J. P. Koch, T. M. Chused, and S. E. Jorgensen. 1962. Utilization of L-α-glycerophosphate by Escherichia coli without hydrolysis. Proc. Nat. Acad. Sci. U. S. A. 48: 2145-2150.

Lin, E. C. C., A. P. Levin, and B. Magasanik. 1960. The effect of aerobic metabolism on the inducible glycerol dehydrogenase of Aerobacter aerogenes. J. Biol. Chem. 235: 1824-1829.

Luria, S. E. 1960. The bacterial protoplasm: composition and organization. p. 13. In I. C. Gunsalus and Roger Y. Stanier (ed), The bacteria, vol. I. Academic Press, Inc., New York.

Malamy, M. H., and B. L. Horecker. 1964. Release of alkaline phosphatase from cells of E. coli upon lysozyme spheroplast formation. Biochem. 3: 1889-1893.

Manno, J. A., and D. Schachter. 1970. Energy coupled influx of thiomethylgalactoside into Escherichia coli. J. Biol. Chem. 245: 1217-1223.

Marquis, R. E. 1965. Nature of the bactericidal action of antimycin A for Bacillus megaterium. J. Bacteriol. 89: 1453-1459.

Mitchell, P. 1962. Molecule, group and electron translocation through natural membranes. Biochem. Soc. Symp. 22: 142-168.

Mitchell, P. 1967. Proton-translocation phosphorylation in mitochondria,chloroplasts and bacteria: natural fuel cells and solar cells. Fed. Proc. 26: 1370.

Mitchell, P., and J. Moyle. 1967. Acid-base titration across the membrane system of rat-liver mitochondria: catalysis by uncouplers. Biochem. J. 104: 588-600.

Mose, L. J. 1970. Metabolic defects and phage resistance in Pseudomonas aeruginosa. M. S. Thesis, Oklahoma State University, Stillwater.

Neish, A. C. 1952. Analytical methods for bacterial fermentations. National Research Council of Canada, Report No. 46-8-3 (2nd ed).

Neu, H. C., and L. A. Heppel. 1964. The release of ribonuclease into the medium when Escherichia coli cells are converted to sphaeroplasts. J. Biol. Chem. 239: 3893-3900.

Newsholme, E. A., J. Robinson, and K. Taylor. 1967. A radiochemical enzymatic activity assay for glycerol kinase and hexokinase. Biochim. Biophys. Acta 132: 338-346.

Nikaido, H. 1962. Phospholipid as a possible component of carrier system in $\beta$-galactoside permease of Escherichia coli. Biochem. Biophys. Res Commun. 9: 486-492.

Paigen, K., and B. Williams. 1970. Catabolite repression and other control mechanisms in carbohydrate utilization. Adv. Microb. Physiol. 4: 251-324.

Pardee, A. B. 1966. Purification and properties of a sulfate-binding protein from Salmonella typhimurium. J. Biol. Chem. 241: 5886-5892.

Pardee, A. B. 1968. Membrane transport proteins. Science 162: 632-637.

Pardee, A. B., and L. S. Prestidge. 1965. Cell-free activity of a sulfate binding site involved in active transport. Proc. Nat. Acad. Sci. U. S. A. 55: 189-191.

Pardee, A. B., L. S. Prestidge, M. B. Whipple, and J. Dreyfuss. 1966. A binding site for sulfate and its relation to sulfate transport into Salmonella typhimurium. J. Biol. Chem. 241: 3962-3969.

Pardee, A. B., and K. Watanabe. 1968. Protein location in bacteria. J. Bacteriol. 96: 1049-1054.

Pavlasova, E., and F. M. Harold. 1969. Energy coupling in the transport of β-galactoside by Escherichia coli: effect of proton conductors. J. Bacteriol. 98: 198-204.

Penrose, W. R., G. E. Nichoalds, J. R. Piperno, and D. L. Oxender. 1968. Purification and properties of a leucine-binding protein from Escherichia coli. J. Biol. Chem. 243: 5921-5928.

Phibbs, P. V., Jr., and R. G. Eagon. 1970. Transport and phosphorylation of glucose, fructose, and mannitol by Pseudomonas aeruginosa. Arch. Biochem. Biophys. 138: 470-482.

Piperno, J. R., and D. L. Oxender. 1966. Amino acid-binding protein released from Escherichia coli by osmotic shock. J. Biol. Chem. 241: 5732-5743.

Piperno, J. R., and D. L. Oxender. 1968. Amino acid transport systems in Escherichia coli K12. J. Biol. Chem. 243: 5914-5920.

Prestidge, L. A., and A. B. Pardee. 1965. A second permease for methyl-thio-β-D-galactoside in Escherichia coli. Biochim. Biophys. Acta 100: 591-593.

Rickenberg, H. V., G. N. Cohen, G. Buttin, and J. Monod. 1956. La galactoside perméase d'Escherichia coli. Ann. Inst. Pasteur 91: 829-857.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1957. Studies of biosynthesis in Escherichia coli. 2nd printing. Carnegie Inst. Wash. Pub. 607.

Rogers, S. W., H. E. Gilleland, Jr., and R. G. Eagon. 1969. Characterization of a protein-lipopolysaccharide complex released from cell walls of Pseudomonas aeruginosa by ethylenediaminetetraacetic acid. Can. J. Microbiol. 15: 743-748.

Roseman, S. 1969. The transport of carbohydrates by a bacterial phosphotransferase system. J. Gen. Physiol. 54: 138-180.

Rosen, B. P. 1971. Basic amino acid transport in Escherichia coli. J. Biol. Chem. 246: 3653-3662.

Rosenberg, T., and W. Wilbrandt. 1957. Uphill transport induced by counterflow. J. Gen. Physiol. 41: 289-296.

Rotman, B. 1959. Separate permeases for the accumulation of methyl-β-D-galactoside and methyl-β-D-thiogalactoside in Escherichia coli. Biochim. Biophys. Acta 32: 599-601.

Rotman, B., A. K. Ganesan, and R. Guzman. 1968. Transport systems for galactose and galactosides in Escherichia coli. II. Substrate and inducer specificities. J. Mol. Biol. 36: 247-260.

Rotman, B., and R. Guzman. 1961. Transport of galactoside from the inside to the outside of Escherichia coli. Pathol. Biol. 9: 806-810.

Rotman, B., and J. Radojkovic. 1964. Galactose transport in Escherichia coli. The mechanism underlying the retention of intracellular galactose. J. Biol. Chem. 239: 3153-3156.

Sanno, Y., T. H. Wilson, and E. C. C. Lin. 1968. Control of permeation to glycerol in cells of Escherichia coli. Biochem. Biophys. Res. Commun. 32: 344-349.

Scarborough, G. A., M. K. Rumley, and E. P. Kennedy. 1968. The function of adenosine-5-triphosphate in the lactose transport system of Escherichia coli. Proc. Nat. Acad. Sci. U. S. A. 60: 951-958.

Schultz, S. G., and P. F. Curran. 1970. Coupled transport of sodium and organic solutes. Physiol. Rev. 50: 637-718.

Shilo, M., and R. Y. Stanier. 1957. The utilization of the tartaric acids by pseudomonads. J. Gen. Microbiol. 16: 482-490.

Simoni, R. D., M. Levinthal, F. D. Kundig, W. Kundig, B. Anderson, P. E. Hartman, and S. Roseman. 1967. Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. Proc. Nat. Acad. Sci. U. S. A. 58: 1963-1970.

Simoni, R. D., M. F. Smith, and S. Roseman. 1968. Resolution of a staphylococcal phosphotransferase system into four protein components and its relation to sugar transport. Biochem. Biophys. Res. Commun. 31: 804-811.

Sistrom, W. R. 1958. On the physical state of the intracellularly accumulated substrates of β-galactoside permease in Escherichia coli. Biochim. Biophys. Acta 29: 579-587.

Skou, J. C. 1965. Enzymatic basis for active transport of $Na^+$ and $K^+$ across cell membrane. Physiol. Rev. 45: 596-617.

Slater, E. C. 1966. Oxidative phosphorylation. p. 327-396. In M. Florkin and E. H. Stotz (ed), Comprehensive biochemistry, vol. 14. American Elsevier Publishing Co., New York.

Smith, L. 1961. Cytochrome systems in aerobic electron transport. p. 365. In I. C. Gunsalus and R. Y. Stanier (ed), The bacteria, vol. II. Academic Press, New York.

Smith, L. 1968. The respiratory chain system of bacteria. p. 73. In T. P. Singer (ed), Biological oxidations. Interscience, New York.

Sowerby, J. M., and J. H. Ottaway. 1966. The enzymatic estimation of glutamate and glutamine. Biochem. J. 99: 246-252.

Stein, W. D. 1967. The movement of molecules across cell membranes. Academic Press, New York.

Stock, J., and S. Roseman. 1971. A sodium-dependent sugar co-transport system in bacteria. Biochem. Biophys. Res. Commun. 44: 132-138.

Stoeber, F. 1957. Sur la $\beta$-glucuronide-permease d'Escherichia coli. Compt. Rend. 244: 1091-1094.

Sutherland, E. W., C. F. Cori, R. Haynes, and N. S. Olson. 1949. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem. 180: 825-837.

Tanaka, S., S. A. Lerner, and E. C. C. Lin. 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J. Bacteriol. 93: 642-648.

Telegdi, M., and T. Keleti. 1964. The role of sulfhydryl groups in $\alpha$-glycerophosphate dehydrogenase (L-$\alpha$-glycerol-3 phosphate:NAD oxidoreductase 1.1.1.8.) activity. Acta Physiol. Acad. Sci. Hung. 25: 181-189.

Tsay, S. S. 1971. Transport and catabolism of glycerol in Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

Vogel, H. J., and R. G. Vogel. 1967. Regulation of protein systhesis. Ann. Rev. Biochem. 36: 519-538.

Wang, R. J., H. G. Morse, and M. L. Morse. 1970. Carbohydrate accumulation and metabolism in Escherichia coli: the characteristics of the reversions of ctr mutations. J. Bacteriol. 104: 1318-1324.

Whittam, R., K. P. Wheeler, and A. Blake. 1964. Oligomycin and active transport reactions of cell membranes. Nature 203: 720-724.

Wilkins, P. O. 1970. Transport and binding of galactose by Streptococcus faecalis. Can. J. Microbiol. 16: 1145-1151.

Wilson, O. H., and J. T. Holden. 1969. Stimulation of arginine transport in osmotically shocked Escherichia coli W cells by purified arginine-binding protein fractions. J. Biol. Chem. 244: 2743-2749.

Winkler, H. H., and T. H. Wilson. 1966. The role of energy coupling in the transport of β-galactosides by Escherichia coli. J. Biol. Chem. 241: 2200-2211.

Wong, P. T. S., and T. H. Wilson. 1970. Counterflow of galactosides in Escherichia coli. Biochim. Biophys. Acta 196: 336-350.

Zierdt, C. H., and P. J. Schmidt. 1964. Dissociation in Pseudomonas aeruginosa. J. Bacteriol. 87: 1003-1010.

Zwaig, N., and E. C. C. Lin. 1966. Feedback inhibition of glycerol kinase, a catabolic enzyme in Escherichia coli. Science 153: 755-757.

# VITA

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