A NONPHOSPHORYLATED PATHWAY FOR METABOLISM OF GLYCEROL BY PSEUDOMONAS AERUGINOSA

By

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A NONPHOSPHORYLATED PATHWAY FOR METABOLISM OF GLYCEROL BY PSEUDOMONAS AERUGINOSA

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CHAPTER I

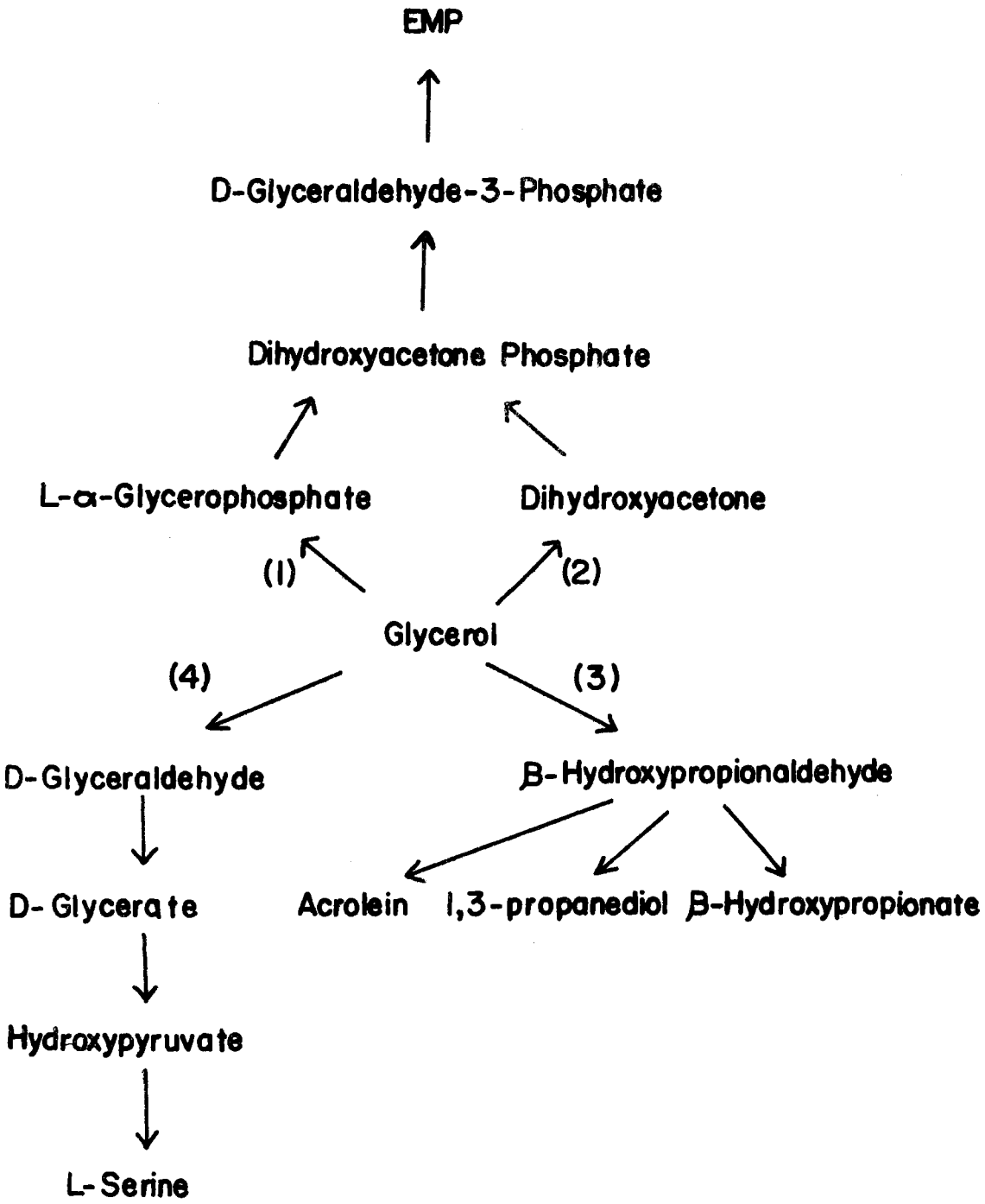
INTRODUCTION

The genus Pseudomonas consists of a group of aerobic, heterotrophic, gram-negative, non-sporeforming, rod-shaped bacteria, which are well known for their metabolic versatility. Stanier et al. (1966) documented the ability of 175 strains of the fluorescent pseudomonads to utilize 146 different organic compounds as their sole sources of carbon and energy. The ability of most strains to grow on the majority of organic compounds tested demonstrates the diverse metabolic capabilities of this group of pseudomonads. Doelle (1969) has recently reviewed the catabolic activities of the Pseudomonadaceae.

One of the organic compounds utilized by 99 per cent of the strains tested by Stanier's group was glycerol. The metabolism of this compound is the main concern of the present study. Figure 1 illustrates the variety of ways that glycerol may be metabolized by bacteria.

The first two pathways for glycerol catabolism shown in Figure 1 appear to be the major ones utilized by bacteria, and the occurrence of these pathways in various species has been reviewed by Ding (1972). In pathway 1, glycerol is phosphorylated by glycerokinase to L-α-glycerophosphate, which in turn is acted on by L-α-glycerophosphate dehydrogenase to form dihydroxyacetone phosphate (Magasanik et al., 1953; Rush et al., 1957). The widespread use of this pathway among bacteria is illustrated by its presence in four unrelated organisms: Acetobacter

Figure 1. Pathways for Glycerol Metabolism


EMP
D-Glyceraldehyde-3-Phosphate
Dihydroxyacetone Phosphate
L-α-Glycerophosphate
Dihydroxyacetone
(1)
(2)
Glycerol
(4)
(3)
D-Glyceraldehyde
β-Hydroxypropionaldehyde
D-Glycerate
Acrolein
1,3-propanediol
β-Hydroxypropionate
Hydroxypyruvate
L-Serine

suboxydans (Hauge et al., 1955), Aerobacter aerogenes (Magasanik et al., 1953; Burton and Kaplan, 1953), Streptococcus faecalis (Gunsalus and Umbreit, 1945), and Mycobacterium smegmatis (Hunter, 1953).

Pathway 2 involves dehydrogenation of glycerol to dihydroxyacetone by a glycerol dehydrogenase (Asnis and Brodie, 1953). The dihydroxyacetone is converted to dihydroxyacetone phosphate by a kinase (Hauge et al., 1955). Representative bacteria that utilize this pathway include A. suboxydans (Hauge et al., 1955), Escherichia coli (Asnis and Brodie, 1953), A. aerogenes (Burton and Kaplan, 1953) and M. tuberculosis (Goldman, 1963).

A third pathway for glycerol dissimilation involves conversion of glycerol to hydroxypropionaldehyde, probably by dehydration (Voisenet, 1918). The hydroxypropionaldehyde is either reduced to 1,3-propanediol, oxidized to hydroxypropionic acid, or converted to acrolein by a spontaneous chemical process (Cantoni and Molnar, 1967).

A fourth pathway for glycerol catabolism, which has not been previously reported in bacteria, was suggested by Brown (1972) as a possible metabolic route in Pseudomonas aeruginosa. Lamanna and Mallette (1965) previously suggested a portion of this pathway as a route for glyceraldehyde catabolism in pseudomonads. Pathway 4 involves a glycerol dehydrogenase which converts glycerol to glyceraldehyde (Brown, 1972). Also proposed were subsequent dehydrogenations of glyceraldehyde and glyceric acid which ultimately form hydroxypyruvic acid (Lamanna and Mallette, 1965; Brown, 1972). An aldehyde dehydrogenase with a broad substrate specificity which also includes activity with glyceraldehyde has been found in P. aeruginosa by Tigerstrom and Razzell (1968). In addition, a hydroxypyruvate reductase which may also function as a glyc-

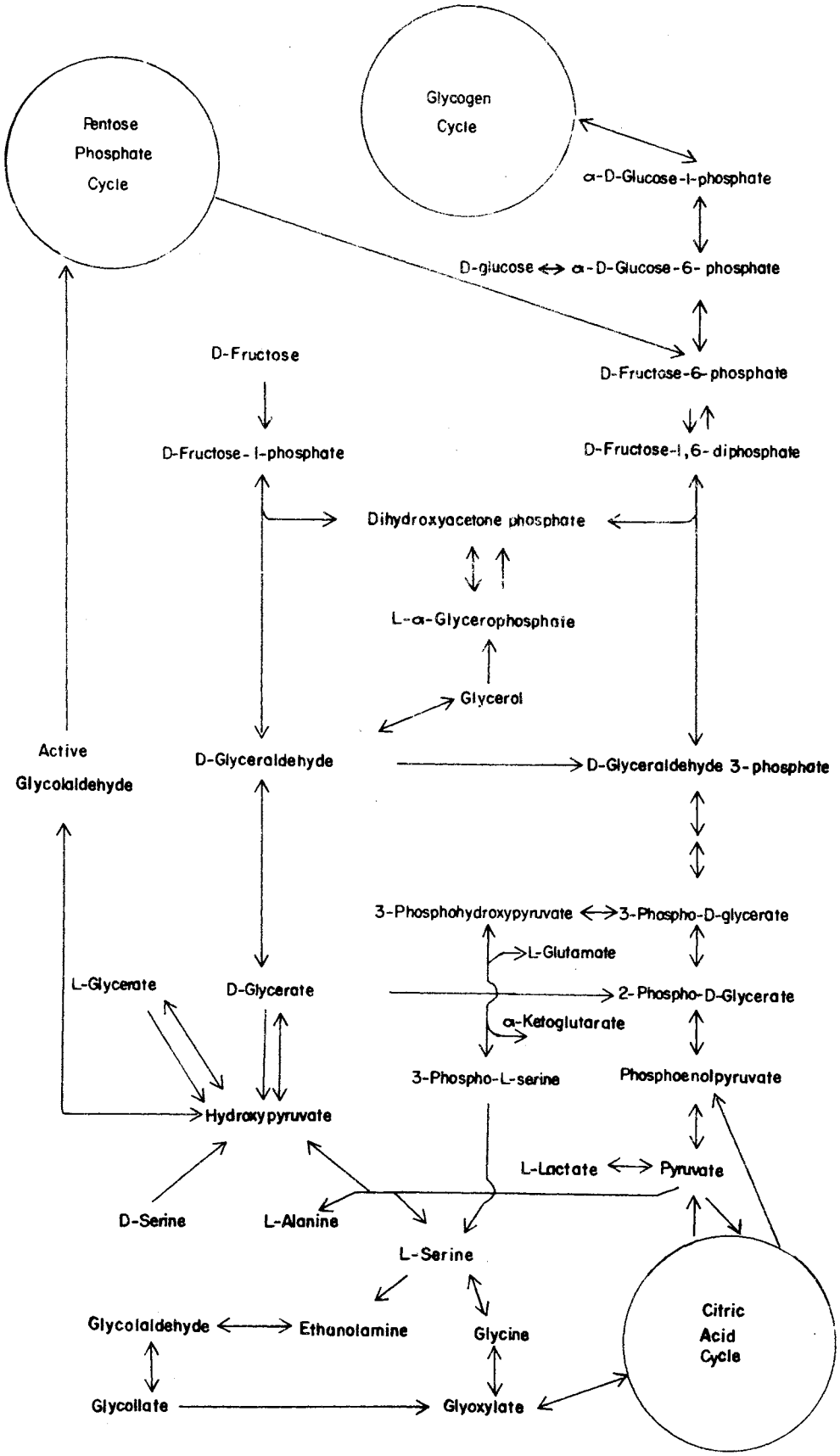
erate dehydrogenase has been found in P. acidovorans (Kohn and Jakoby, 1968). The hydroxypyruvic acid produced by pathway 4 could be aminated to serine by a transaminase. This pathway is commonly found in the liver of many mammals. Figure 2 shows the relationship of this pathway to other reactions of intermediary metabolism in guinea pig liver as presented by Heinz et al. (1962).

Since more than one of the above pathways can be found in the same organism, it is important to determine the conditions under which a given pathway may be used. Kistler and Lin (1971) were able to show with E. coli that the first pathway may be used under either aerobic or anaerobic conditions, although the dehydrogenases are not identical. A pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase was utilized under aerobic conditions and a flavin-linked glycerophosphate dehydrogenase under anaerobic conditions. The anaerobic pathway would only function in the presence of an added electron acceptor such as nitrate or fumarate. Lin et al. (1960) found that A. aerogenes may catabolize glycerol anaerobically by an inducible, diphosphopyridine nucleotide-linked dehydrogenase and an inducible dihydroxyacetone kinase without added electron acceptors. The above pathway did not function in A. aerogenes grown under aerobic conditions, where glycerol was metabolized by the first pathway.

In the third pathway, E. freundii utilizing glycerol as its sole carbon source has been shown to ferment glycerol anaerobically, producing a glycerol intermediate, hydroxypropionaldehyde, which serves as an electron acceptor to form 1,3-propanediol (Mickelson and Werkman, 1940). No other electron acceptors were necessary.

In Brown's proposed pathway, there are three oxidative steps that

Figure 2. Reactions of Intermediary Metabolism Related to Metabolism of Glycerol (Heinz, et al., 1962)


Pentose
Phosphate
Cycle
Glycogen
Cycle
α-D-Glucose-1-phosphate
D-glucose ↔ α-D-Glucose-6- phosphate
D-Fructose
D-Fructose-6-phosphate
D-Fructose-1-phosphate
D-Fructose-1,6-diphosphate
Dihydroxyacetone phosphate
L-α-Glycerophosphate
Glycerol
Active
Glycolaldehyde
D-Glyceraldehyde
D-Glyceraldehyde 3-phosphate
3-Phosphohydroxypyruvate ↔ 3-Phospho-D-glycerate
L-Glutamate
L-Glycerate
D-Glycerate
2-Phospho-D-Glycerate
α-Ketoglutarate
3-Phospho-L-serine
Phosphoenolpyruvate
Hydroxypyruvate
L-Lactate ↔ Pyruvate
D-Serine
L-Alanine
L-Serine
Glycolaldehyde ↔ Ethanolamine
Glycine
Citric
Acid
Cycle
Glycollate
Glyoxylate

occur in sequence; this would appear to require aerobic conditions. All work with potential enzymes in this pathway has been done only with cells grown under aerobic conditions (Brown, 1972; Tigerstrom and Razzell, 1968; Kohn and Jakoby, 1968).

The regulation of uptake and dissimilation of glycerol in bacteria is best understood in E. coli. In 1964, Koch et al. were able to show induction of glycerol kinase, L-α-glycerophosphate dehydrogenase and the L-α-glycerophosphate transport system by growth of E. coli on either glycerol or L-α-glycerophosphate. These three factors are responsible for the initial steps in dissimilation of glycerol and L-α-glycerophosphate according to pathway 1, Figure 1. In mutants lacking glycerol kinase, L-α-glycerophosphate, but not glycerol, induced formation of the transport system and the dehydrogenase. It was proposed that the three factors belonged to different operons which were under the control of the same regulator gene, since a single mutation resulted in constitutivity for all three and each showed a different sensitivity to catabolite repression. The above type of organization for genetic control is termed a regulon (Maas and Clark, 1964). Here structural genes found in different operons are under simultaneous control by a single regulator gene, while in the classic operon model for genetic control, the structural genes are under the control of a single operator gene which in turn is under the control of a regulator gene (Jacob and Monod, 1961).

Hayashi and Lin (1965a) found that mutants lacking glycerol kinase were unable to take up ^{14}C-glycerol. They also found a glycerol kinase-deficient mutant in which glycerophosphate could induce a protein immunologically indistinguishable from glycerol kinase while glycerol failed to induce (Hayashi and Lin, 1965b). Finally it was concluded that

L-α-glycerophosphate was the inducer which interacted with the product of the regulator gene of the glycerophosphate (glp) regulon to induce formation of the glp proteins.

Glycerophosphate is taken up by active transport in E. coli, as indicated by the ability of sodium azide and 2,4-dinitrophenol to prevent its uptake (Hayashi et al., 1964). A mutant lacking the L-α-glycerophosphate transport system was still able to grow on glycerol. Sanno et al. (1968) concluded that glycerol was taken up by facilitated diffusion under the control of the glp regulon. As measured by following the rate of change in cell volume upon exposure to a hypertonic solution of glycerol, glycerol- and glycerophosphate-grown cells demonstrated increased permeability to glycerol as compared to glucose-grown cells. Also the increased permeability to glycerol could not be induced by glycerol in glycerol kinase-deficient mutants, but could be induced in these mutants by glycerophosphate.

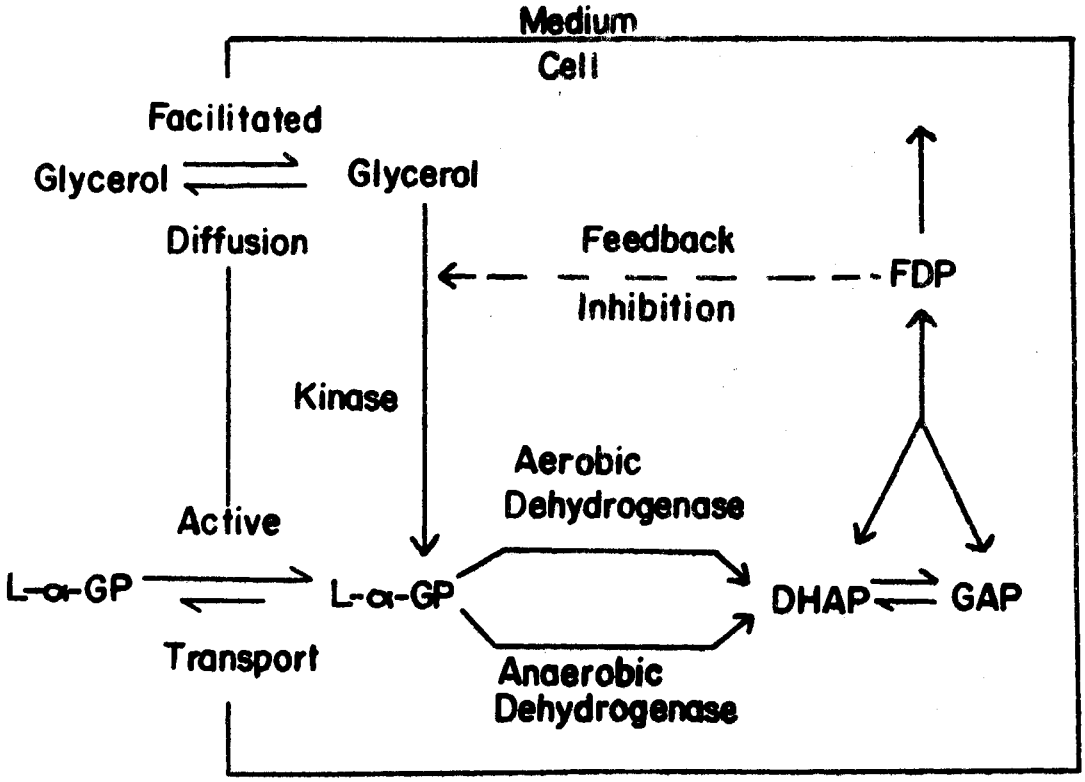
Feedback inhibition of glycerol kinase by fructose-1,6-diphosphate was found to exclude glycerol dissimilation during glucose metabolism (Zwaig and Lin, 1966). A glycerol kinase mutant of E. coli resistant to inhibition by fructose-1,6-diphosphate has been shown to grow faster on glycerol than does the wild type (Zwaig et al., 1970). Constitutive mutants of E. coli of the above type produced a bactericidal factor, methylglyoxal, from glycerol (Freedberg et al., 1971). Figure 3 depicts the pathways for glycerol and L-α-glycerophosphate dissimilation in E. coli as determined by Lin's research group (Berman-Kurtz et al., 1971).

Kistler and Lin (1971) concluded that pathway 2 in Figure 1 was not used in dissimilation of glycerol in E. coli. They based their conclusions on the nonspecific induction of the NAD-linked glycerol dehydro-

Figure 3. Pathways for Glycerol and L-α-Glycerophosphate Dissimilation in Escherichia coli (Berman-Kurtz et al., 1971).

Abbreviations:

DHAP, Dihydroxyacetone phosphate
FDP, D-Fructose-1,6-diphosphate
GAP, D-Glyceraldehyde-3-phosphate
L-α-GP, L-α-Glycerophosphate


Medium
Cell
Facilitated
Glycerol
Glycerol
Diffusion
Feedback
Inhibition
FDP
Kinase
Aerobic
Dehydrogenase
Active
L-α-GP
L-α-GP
DHAP
GAP
Transport
Anaerobic
Dehydrogenase

genase as well as its unresponsiveness to mutation in the glp regulator gene. They felt that it probably played a role in glycerol production in certain fermentations.

The regulation of uptake and dissimilation of glycerol in P. aeruginosa has been the subject of investigation in this laboratory since 1966. In 1968, Cowen reported the inducibility of glycerol catabolism in this organism, and the presence of glycerol kinase and L-α-glycerophosphate dehydrogenase. L-α-glycerophosphate could not be taken up as it is in E. coli. Tsay (1971) investigated further the conditions for induction of the above enzymes. Indirect evidence indicated that L-α-glycerophosphate might be the inducer of these enzymes in P. aeruginosa. The enzymes of pathway 1, Figure 1, and the glycerol transport system were shown not to be subject to catabolite repression in the presence of glucose.

A glycerol binding protein present in shock fluids of cells capable of taking up glycerol but absent in transport-negative mutants was found by Tsay (1971). Brown (1972) presented evidence to indicate active transport of glycerol involving a glycerol-specific binding protein. Glycerol binding and transport activities were found to be completely inhibited by sodium azide and sodium arsenate while no effect on glycerol kinase or L-α-glycerophosphate dehydrogenase occurred. The fact that binding, as well as transport, was inhibited negated the use of these inhibitors to provide evidence for active transport. However, 2,4-dinitrophenol inhibited transport without affecting binding or either of the enzyme activities. Therefore, it was concluded that transport of glycerol in P. aeruginosa is probably energy-dependent. Also, it was shown that glycerol kinase activity was not directly involved in uptake of

glycerol, as it has been stated to be in E. coli (Lin, 1970), since a mutant with full transport ability had no glycerol kinase activity and another mutant unable to transport glycerol had high glycerol kinase activity. Growth on glycerol of wild-type P. aeruginosa induced full glycerol transport activity while growth on glucose and lactate induced partial transport activity.

Additional information regarding the relationship of dissimilation of glycerol to glucose metabolism was obtained by Heath (1971) in this laboratory. He found that glycerol induced the glucose catabolic enzymes in P. aeruginosa. The fact that many mutants which failed to grow on glucose also failed to grow on glycerol suggested that glycerol metabolism may depend on some of the reactions of glucose metabolism. It was shown that the failure to grow on these two compounds was due to a single lesion and that enzymes required for glycerol catabolism were present in the mutants. He proposed the following model to explain the dependence of glycerol metabolism on glucose metabolism. Glycerol is metabolized to glyceraldehyde-3-phosphate and conversion of this intermediate requires a NAD^+-linked glyceraldehyde-3-phosphate dehydrogenase which is induced by glucose-6-phosphate. The latter compound would have to be produced from glycerol, which would thus induce some of the glucose catabolic enzymes.

Work by other research groups serves to confirm and extend the work of Heath. Glycerol and glycerate were found to induce high levels of hexose-catabolizing enzymes in P. aeruginosa (Hyeleman and Phibbs, 1972). They concluded that some intermediate of glucose catabolism, probably a triose phosphate, was the actual inducer. Lessie and Neidhardt (1967) had previously reported that the highest levels of the Entner-Doudoroff

pathway enzymes were induced by growth on glycerol.

Very little is known about the regulation of glycerol catabolism by pathway 3 (Figure 1). It has been noted, however, that lactobacilli which cannot grow on glycerol alone produce nearly twice as much growth on glucose plus glycerol as on glucose alone (Sobolov and Smiley, 1960). Lactobacilli grown on glucose plus glycerol produce much more 1,3-propanediol from glycerol than when grown on glycerol alone. Also, under these same conditions much less ethanol is produced as compared with growth on only glucose. This led to the conclusion that reduction of β-hydroxypropionaldehyde to 1,3-propanediol was preferred to reduction of acetyl phosphate to ethanol. The acetyl phosphate yields adenosine triphosphate, doubling the energy yield of the fermentation and accounting for the increased growth on glucose plus glycerol.

Knowledge of the regulation of glycerol catabolism by pathway 4 (Figure 1) is lacking. In the liver of mammals where the pathway is known to be present (Heinz et al., 1962), all of the enzymes in the pathway are reversible and could play a role in glycerol dissimilation and gluconeogenesis from L-serine and a variety of other intermediates.

As noted previously, there are two enzymes found in different species of Pseudomonas which could function in this pathway. Tigerstrom and Razzell (1968) proposed that their aldehyde dehydrogenase from P. aeruginosa, which is capable of oxidizing glyceraldehyde, was paraconstitutive since it was present in low levels in cells grown on glucose, acetate or glycerol and in very high levels in cells grown on ethanol or ethylene glycol. The hydroxypyruvate reductase (glycerate dehydrogenase) of P. acidovorans was inhibited by dihydroxyfumarate, an intermediate in glyoxylate metabolism (Kohn and Jakoby, 1968).

Beef liver glycerate dehydrogenase has been found to be sensitive to the concentrations and relative proportions of NAD^+ and $NADP^+$ (Rosenblum et al., 1971). High concentrations of NAD^+ enhanced activity while high concentrations of $NADP^+$ inhibited activity. Adenosine triphosphate, guanosine triphosphate and phosphohydroxypyruvate inhibited glycerate dehydrogenase. The adenosine triphosphate was not competitive with either $NADH_2$ or hydroxypyruvate, and phosphohydroxypyruvate was not competitive with hydroxypyruvate. It was concluded that phosphohydroxpyruvate, a metabolite in the phosphorylated pathway, could inhibit the activity of an enzyme in the nonphosphorylated pathway thereby determining which pathway would be functioning at any given time. Sugimoto et al. (1972) found that glycolytic intermediates such as pyruvate, α-D-fructose-1,6-diphosphate, 3-phospho-D-glycerate, 2,3-diphospho-D-glycerate, and 2-phospho-D-glycerate as well as adenosine triphosphate inhibited beef liver glycerate dehydrogenase. They concluded that catabolic conversion of L-serine to glycolytic intermediates probably occurred via glyceric acid. These intermediates would serve a regulatory function as feedback inhibitors.

It was noted by Brown (1972) that glycerol kinaseless mutants of P. aeruginosa, unable to grow on glycerol, rapidly produced $^{14}CO_2$ from U-^{14}C-glycerol. Cells grown in medium containing both glycerol and glucose produced $^{14}CO_2$ approximately three times as rapidly as did cells grown on glucose alone. No known decarboxylation reactions in carbohydrate metabolism could explain this rapid production of radioactive carbon dioxide. Bell et al. (1971) found that the decarboxylation of phosphatidylserine to phosphatidylethanolamine occurred in E. coli and Salmonella typhimurium. Brown proposed that this reaction could explain

the rapid release of radioactive carbon dioxide if glycerol was metabolized to serine via pathway 4 in Figure 1.

The present investigation was undertaken to determine if the pathway for glycerol dissimilation proposed by Brown is present in P. aeruginosa, strain PA-1. This involved confirmation of the presence of glycerol dehydrogenase, glyceraldehyde dehydrogenase (aldehyde dehydrogenase), and glycerate dehydrogenase in cells grown on glycerol. Regulatory mechanisms involving the proposed pathway were also to be examined. This seemed important since very little is known about the regulation of the nonphosphorylated pathway for dissimilation of glycerol in animals or bacteria.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains

Pseudomonas aeruginosa, strain 1, (designated PA-1) was originally obtained from Dr. B. W. Holloway, Monash University, Clayton, Australia. A mutant of PA-1 unable to utilize glycerol as a sole carbon source was isolated after nitrosoguanidine treatment by Dr. Floyd E. White, Jr. (1972) and designated GA-73.

Media

Minimal medium A was a modification of Roberts' M-9 medium (Roberts et al., 1957) and contained per liter of solution: NH_4Cl, 1.0 g; $Na_2HPO_4 \cdot 7H_2O$, 11.3 g; KH_2PO_4, 3.0 g; NaCl, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; and $FeSO_4$, 0.5 mg. The $FeSO_4$ and $MgSO_4 \cdot 7H_2O$ were sterilized separately as 0.1 per cent and 40 per cent solutions (w/v), respectively. Carbon sources which were autoclaved separately as 25 per cent solutions (w/v) included: sodium lactate, sodium succinate, glycerol, and glucose. Ethanol and glyceric acid were filter-sterilized as 25 per cent solutions (w/v) using 47 mm Gelman filters, HA 0.45 μm pore size. All carbon sources were added aseptically to sterile minimal medium A to give a final concentration of 0.5 per cent. Minimal medium A was always sterilized in bottles and aseptically transferred to sterile flasks or test

tubes after all components sterilized separately were added.

Nutrient agar was prepared using Difco nutrient agar plus enough Bacto-agar (Difco) to give a final concentration of 2.0 per cent agar. Plates containing 30 ml each of nutrient agar were dried for 24 hr at 37°C before use.

Cultivation of Bacteria

Stock cultures of P. aeruginosa strains were transferred to new nutrient agar slants once each month and two drops of sterile 25 per cent glycerol were aseptically added to the surface of each slant. The inoculated slants were incubated at 37°C for 12-18 hours and stored at 4°C.

For most experiments cells were grown in minimal medium A plus the desired carbon source. Cells were grown in either Erlenmeyer or Fernbach flasks on a reciprocal shaker at 37°C. Growth of the cultures was measured by determining their optical density at 540 nm in an 18 mm diameter tube on a Coleman Junior Spectrophotometer, Model D. When cells were to be harvested, they were harvested by centrifugation when the cultures had reached an optical density of 0.45-0.55. At the time of harvesting, each culture was checked for purity by streaking a nutrient agar plate. Cells which were to be used for induction of enzymes were harvested at room temperature and those to be used for immediate preparation of cell-free extracts at 4°C.

P. aeruginosa mutants which could not use glycerol as a sole carbon source were checked before use by measuring growth with different carbon sources. Tubes 18 mm in diameter and containing 6 ml of minimal medium A plus the desired carbon source were inoculated with 0.05 ml of a cell suspension. The cell suspensions used for inocula were obtained by

aseptically adding 2.5 ml of minimal medium A to an overnight culture on a nutrient agar slant followed by suspension of the cells with an inoculation loop. The tubes were incubated at 37°C on a reciprocal shaker for 24 hours and their optical densities read at 540 nm.

Preparation of Cell-free Extracts

Cells were grown with shaking in 200 ml of minimal medium A plus 0.5 per cent of each desired carbon source in a 2 l Fernbach flask, harvested by centrifugation at 12,500 x g for 10 minutes in a refrigerated centrifuge, and washed twice with 0.85 per cent (w/v) saline. The resultant pellets were either used immediately or maintained frozen at -20°C for not more than two weeks.

Frozen or fresh pellets were suspended in 5 ml of cold 0.02 M Tris buffer, pH 8.5, using a Vortex Junior Mixer. The cells were broken by sonication with a Bronson sonifier using 15 second bursts and 60 second cooling periods between bursts. The cells were sonicated in 10 ml Calbiochem MiniBeakers with the beaker being placed in crushed ice during sonication and in an ice water bath during cooling periods. Cells were subjected to as many sonic bursts as required to produce clearing of the cell suspension.

Whole cells and cell debris were removed by centrifugation at 12,500 x g for 10 minutes in a refrigerated centrifuge. The supernatant (cell-free extract) was decanted, placed in ice, and used immediately for enzyme assays. A 1:20 dilution of the cell-free extract was also immediately prepared in 0.02 M Tris buffer, pH 8.5, and frozen for later use in protein determinations.

Enzyme Assays

All enzyme assays were carried out spectrophotometrically with a Cary 14 dual beam recording spectrophotometer. No more than a total volume of 1.2 ml of liquid in a 1.5 ml quartz cuvette was used in an assay. The contents of the two cuvettes differed only in the substitution of distilled water for substrate in the blank except where otherwise noted. Components for each enzyme assay were added in the order listed, with components mixed by inversion prior to addition of cell-free extract and again following the addition of the component added to start the reaction. All assays were carried out at room temperature with buffer solutions at room temperature and all other components at $0^{o}C$.

The reduction or oxidation of pyridine nucleotides was used to follow enzyme activity by observing the change in optical density at 340 nm. When the oxidation of pyridine nucleotides was to be followed, the positions of the blank and reaction mixture were reversed, except where otherwise noted, so an increase in optical density was observed.

A unit of enzyme was defined as that amount required to oxidize or reduce one nanomole of pyridine nucleotide per minute under the specified conditions. A millimolar extinction coefficient of 6.2 (Dawson et al., 1959) at 340 nm for reduced pyridine nucleotide was used to calculate all enzyme activities.

Assays described below are modifications of enzyme assays which have previously been reported for P. aeruginosa. All enzyme assays developed during the course of this study will be presented in the following chapter.

Glycerol Dehydrogenase

An assay for glycerol dehydrogenase previously developed by Goldman (1963) was modified by Brown (1972), but the conditions of the assay for enzyme from P. aeruginosa were not optimized. The following assay is a further modification of Brown's procedure which optimizes activity of the enzyme from P. aeruginosa. The reaction cuvette contained: 0.5 ml of 0.1 M sodium carbonate buffer, pH 11.0; 0.1 ml of 0.02 M NADP; 0.3 ml of cell-free extract; and 0.1 ml of either 1 M or 80 per cent glycerol. The 80 per cent glycerol gave higher levels of activity and was used when low levels of enzyme were difficult to detect. Since this high concentration of glycerol was difficult to pipet accurately due to its viscosity, it was not used in most instances where easily detectable levels of enzyme were present.

Aldehyde Dehydrogenase

The procedure of Tigerstrom and Razzell (1968) was used to measure aldehyde dehydrogenase activity in ethanol-grown cells with only minor modifications. The reaction cuvette contained: 0.4 ml of 0.2 M sodium phosphate buffer, pH 7.5; 0.1 ml of 0.02 M NAD; 0.1 ml of 1 M mercaptoethanol; 0.1 ml of 1 M potassium chloride; 0.1 ml of cell-free extract and 0.3 ml of 0.1 M glyceraldehyde. The reaction mixture was preincubated for 5 minutes at room temperature prior to addition of substrate. The final pH of the reaction mixture was 7.2, the same as Tigerstrom and Razzell's.

The following assay, which was developed for use with glycerol-grown cells, is a considerable modification of the one developed by Tigerstrom and Razzell (1968). The reaction cuvette contained: 0.4 ml of 0.1 M

Tris buffer, pH 9.1; 0.1 ml of 0.02 M NAD; 0.1 ml of 1 M 2-mercaptoethanol; 0.1 ml of cell free extract; and 0.3 ml of 0.1 M glyceraldehyde.

Determination of the pH Optimum for Enzyme Activity

The assay procedure used to determine the pH optimum for an enzyme is given either in this chapter or in the following one. The buffer used in the assay procedure was usually the major buffer used to determine the pH optimum. When a different buffer was required because of the broad pH range of an enzyme, enzyme activity was determined at the same pH using both buffers to detect any difference in enzyme activity due to buffer. Only buffers giving the same enzyme activity at the overlapping pH were used.

Before determination of each pH optimum, buffer solutions at different pH values covering the desired range were prepared. In general, buffer at a known pH was mixed with all other components of the assay mixture except substrate and the actual pH of the mixture was measured. The pH of the substrate was then adjusted to the pH of the assay mixture just prior to addition of substrate to start the reaction. When a more precise determination of pH optimum was needed, the pH of the assay mixture without substrate was adjusted to the exact value desired using hydrochloric acid or phosphoric acid and sodium hydroxide or potassium hydroxide. When a more alkaline pH than the most alkaline buffer available was necessary, this buffer plus all assay components except substrate were mixed and the pH was adjusted using the appropriate concentration of sodium hydroxide. Finally, the assay mixture without sub-

strate was transferred to two cuvettes in the volume indicated by the assay procedure followed by addition of water to the blank cuvette and substrate (adjusted to the pH of the assay mixture) to the reaction cuvette. At the end of the assay, the contents of the blank and reaction cuvettes were mixed in a 10 ml beaker and the pH was again checked to confirm the initial pH measurement.

A Coleman Model 39 pH meter was used to determine the pH of all assay mixtures minus substrate, substrates, and completed reaction mixtures. Usually a 2-ml volume of liquid in a 10 ml Pyrex beaker was sufficient to measure the pH accurately on this pH meter.

Protein Determination

The amount of protein in cell-free extracts was determined by the procedure of Sutherland et al. (1949). One-tenth milliliter of cell-free extract was diluted 1:20 with 0.02 M Tris buffer, pH 8.5, and either stored frozen at -20°C or used immediately for protein assay. Aliquots of the diluted cell-free extract were made up to 1.0 ml with 0.02 Tris buffer, pH 8.5. Addition of 5 ml of a reagent consisting of 1.0 ml of 4 per cent sodium potassium tartrate, 1.0 ml of 2 per cent $CuSO_4 \cdot 5H_2O$, and 100 ml of 4 per cent Na_2CO_3, mixed just prior to use, was followed by a 40 minute incubation period at room temperature. One-half milliliter of a two-fold water dilution of 2 N phenol reagent (Fisher Scientific Company) was added to each tube and mixed immediately with a Vortex Junior Mixer. After 15 minutes at room temperature, the optical density of each sample was determined at 660 nm.

A water blank and a set of standards containing 30, 60, 90, 120, and 150 µg per ml of bovine serum albumin were included with each assay.

A stock solution of bovine serum albumin, 300 µg per ml, was prepared in 0.02 M Tris buffer, pH 8.5, and diluted with the buffer for standards to compensate for color formation due to the buffer used to dilute cell-free extracts (Gregory and Sajdera, 1970).

Estimation of Aliphatic Aldehydes

The 3-methyl-2-benzothiazolone hydrazone test for aliphatic aldehydes is a colorimetric procedure developed by Sawicki et al. (1961). In this study a modification developed by Bradbury and Jakoby (1971) was used.

To 0.5 ml of sample in an 18 mm diameter tube, 0.5 ml of 0.4 per cent aqueous 3-methyl-2-benzothiazolone hydrazone was added and allowed to react for 3 minutes in a boiling water bath. After cooling the tubes in tap water, 2.5 ml of 0.2 per cent aqueous ferric chloride were added. Following 5 minutes incubation at room temperature, 6.5 ml of acetone were added to each tube to bring the total volume to 10 ml. The absorbance of each tube was then determined at 662 nm using a water sample treated as described above as a blank. A standard curve using glyceraldehyde as the aliphatic aldehyde was included with each determination.

Chromatographic Techniques

Descending Paper Chromatography

Whatman #4 chromatography paper 1 1/2 inches wide and 60 cm long was used for all descending paper chromatography. Samples of standards and unknowns were spotted in the center of the strips with the origin 7 cm from one end. When large volumes of reaction mixtures containing radioactive compounds were used, the sample was applied along the origin

line to within 0.5 cm of both edges of the strip.

The strip chromatograms were developed in 12 x 12 x 24 inch Pyrex chromatography chambers which had been equilibrated for at least 12 hours with the desired solvent system. The chromatograms were allowed to develop until the solvent front reached approximately 10 cm from the bottom of the strip; the solvent front was marked and the strips were air dried.

Spots on chromatograms were detected using the periodic acid-benzidine system of Gordon et al. (1956). A 0.1 M aqueous solution of periodic acid was stored at 4°C and diluted 1 to 20 with acetone just prior to pipetting to saturate a paper strip (approximately 5 ml per strip). After the strip had been air dried, approximately 5 ml of 0.01 M benzidine solution (in a mixture of 0.6 ml of glacial acetic acid, 4.4 ml of distilled water and 95 ml of acetone) was pipetted on to the strip. As the strip dried, compounds chromatographed appeared as white spots in a blue field.

Radioactive bands or spots on paper strips were located using a Packard Radiochromatogram Scanner Model 7201. Initially scans were made at maximum speed and the appropriate linear range. Radiochromatograms of interest were scanned more accurately by varying the window size and scanning speed as recommended by Wood (1968). All scans were repeated at least two times.

Thin-layer Chromatography

Quanta/Gram silica gel thin-layer plates (Quantum Industries) 5 cm wide were used in all thin-layer chromatography. The sample was spotted 2 cm from one end of the plate with no more than 4 samples spotted per plate at one cm intervals. Large volumes applied along the origin were

treated in the same manner as in descending paper chromatography.

The plates were developed in Desaga rectangular chambers which were equilibrated with the appropriate solvent systems for at least 4 hours prior to use. The plates were allowed to develop until the solvent front was within 4 cm of the top of the plate. The solvent front was marked and the plate air-dried.

Radiochromatograms were scanned as described above for descending paper chromatography.

Purification of D-glyceraldehyde-U-^{14}C

D-glyceraldehyde-U-^{14}C obtained from New England Nuclear was approximately 80 per cent pure and was purified by a modification of the procedure of Veneziale (1972). Approximately 10 µcuries of D-glyceraldehyde-U-^{14}C were spotted on a Quantum/Gram silica gel thin-layer plate along the origin line 2 cm from the bottom and 0.5 cm from either edge using a graduated 25 µl lambda pipet and a Clay-Adams suction apparatus. Separation of the D-glyceraldehyde-U-^{14}C from radioactive contaminants was achieved by chromatography using a solvent system of methylene chloride-methanol-0.01 N hydrochloric acid (40:20:3, v:v:v). Non-radioactive D-glyceraldehyde (5 µmoles) was chromatographed on another plate at the same time to identify the radioactive peak which represented D-glyceraldehyde-U-^{14}C. The radioactive contaminants and D-glyceraldehyde were located by use of the Packard Radiochromatogram Scanner Model 7201. The non-radioactive D-glyceraldehyde was detected by spraying with 50 per cent sulfuric acid followed by heating in an oven at 100°C until charring occurred and spots became visible.

The silica gel from the center 2 cm of the D-glyceraldehyde-U-^{14}C

peak was scraped off into a 15 ml conical centrifuge tube using a stainless steel spatula. The D-glyceraldehyde-U-^{14}C was extracted from the silica gel using 1 ml of glass distilled water. After suspension of the silical gel in the water for approximately 5 minutes, it was sedimented by centrifugation in an International Clinical Centrifuge Model CL. The supernatant was carefully removed with a Pasteur pipet and transferred to another 15 ml conical centrifuge tube.

A portion (100 µl) of the supernatant was spotted on another silica gel thin-layer plate, rechromatographed and scanned as previously described. If there was only one radioactive peak and it had the same R_f as non-radioactive D-glyceraldehyde, the D-glyceraldehyde-U-^{14}C was considered pure. If more than one radicactive peak was present, the purification procedure was repeated with the newly concentrated, partially purified D-glyceraldehyde-U-^{14}C. The other portion of the supernatant was concentrated by slanting the conical centrifuge tube in a petri dish which in turn was placed in a vacuum desiccator over concentrated sulfuric acid. Desiccation under vacuum was continued until the supernatant was concentrated to 0.1 ml; it was then refrigerated.

Preparation and Detection of Semicarbazone Derivatives

Since semicarbazone formation is optimal near neutrality (Shriner et al., 1962), solutions of semicarbazide hydrochloride and compounds to be derivatized for standards were adjusted to pH 7-8. For preparation of derivatives of standards, 0.3 ml of a 1 M solution of compound or an equivalent amount of compound at a lower molarity was mixed with an equal volume of semicarbazide hydrochloride solution.

When semicarbazide hydrochloride was used to trap products of enzyme action, the reaction was carried out in the same pH range (7-8). In all enzyme reaction mixtures, 0.1 ml of 0.2 M semicarbazide hydrochloride was added per milliliter of reaction mixture.

The semicarbazide trapping of enzyme products was accomplished by the procedure of Cartwright et al. (1971). All samples were mixed with the appropriate volume of semicarbazide hydrochloride and incubated at room temperature for 30 minutes. At the end of the incubation period, mixtures requiring deproteinization were heated for 10 minutes in a water bath at 100°C and the denatured protein sedimented by centrifugation at 12,500 x g at room temperature. The supernatant was transferred to a test tube by Pasteur pipet and extracted 4 times with 2.5 ml of diethyl ether each time. The ether fractions for each sample were combined and evaporated to dryness.

One-tenth milliliter of ether was added to each tube and the resultant solution of semicarbazone was spotted on a Quanta/Gram thin-layer plate. The thin-layer plates were developed in tertiary amyl alcohol-aqueous ammonia (sp. gr. 0.90)-n-propanol (65:35:5, v:v:v). The semicarbazones on the developed thin-layer plates were detected by spraying with 0.4 per cent 2,4-dinitrophenylhydrazine in 2 N HCl (Dawson et al., 1969). The semicarbazones show up as yellow spots due to their conversion to 2,4-dinitrophenylhydrazones.

Chemicals

Cinnamaldehyde, n-butyraldehyde and iso-butyraldehyde were generously supplied by Dr. E. J. Eisenbraun, Department of Chemistry, Oklahoma State University. Reagent grade acetone was obtained from Merck and

Company. Periodic acid was produced by the G. Frederick Smith Chemical Company. Absolute ethanol was a product of the U.S. Industrial Chemical Company. D-glyeraldehyde and L-serine were purchased from Schwartz-Mann. New England Nuclear was the source of uniformly labelled D-glyceraldehyde-^{14}C.

Chemicals purchased from Fisher Scientific Company (Certified A.C.S. grade) included the following: ammonium chloride, benzaldehyde, benzidine dihydrochloride, n-butanol, ferrous sulfate, glycerol, glycollic acid, glyoxal, isopropanol, magnesium sulfate, mannitol, potassium chloride, potassium hydroxide, semicarbazide hydrochloride, sodium bicarbonate, sodium carbonate, sodium lactate (60 per cent syrup) and sulfuric acid.

Absolute ether, β-hydroxybutyrate, potassium phosphate (monobasic), and sodium butyrate were obtained from Matheson, Coleman and Bell. The following chemicals were produced from K and K laboratories: DL-glyceric acid (65 per cent solution), N-methylbenzothiazolone hydrazone hydrochloride, 3-quinolylhydrazine and ribitol. The Mallinckrodt Company was the source of glacial acetic acid, methylene chloride, and sodium chloride.

J. T. Baker chemicals (Analyzed Reagent grade) included: cupric sulfate (pentahydrate), dextrose, ferric chloride, methanol, potassium sodium tartrate, n-propanol, pyridine, sodium hydroxide and sodium phosphate (dibasic). Bovine serum albumin, i-erythritol, sodium glyoxylate, and triphosphopyridine nucleotide ($NADP^+$) were from the Sigma Chemical Company.

Chemicals purchased from Calbiochem included: dihydroxyacetone dimer, dihydronicotinamide adenine dinucleotide ($NADH_2$), dihydronicotin-

amide adenine dinucleotide phosphate ($NADPH_2$), tris (hydroxymethyl) aminomethane (Tris), and uniformly labelled ^{14}C-glyceric acid and ^{14}C-glycerol. Nicotinamide adenine dinucleotide (NAD^+) was obtained from either Calbiochem or Nutritional Biochemicals. Other chemicals obtained from Nutritional Biochemicals included: DL-glyceric acid (calcium salt), hydroxypyruvic acid (lithium salt), and sodium pyruvate. Eastman Organic Chemicals produced the acetaldehyde, 2-mercaptoethanol, 1,2-propanediol, propionaldehyde and succinic acid (disodium salt).

CHAPTER III

EXPERIMENTAL RESULTS

Enzymes of a Nonphosphorylated Pathway for Glycerol Metabolism

In 1972 Brown, of this laboratory, found preliminary evidence that, in P. aeruginosa, glycerol may be oxidized by a glycerol dehydrogenase producing D-glyceraldehyde. Initially, verification of the presence of such an enzyme and of its end product was necessary in order to proceed with the assumption that a nonphosphorylated pathway for glycerol metabolism did exist in P. aeruginosa.

Glycerol Dehydrogenase

When P. aeruginosa strain PA-1 was grown with glycerol as the sole carbon source and examined for glycerol dehydrogenase activity as described in Chapter II, the enzyme was readily detectable. In Table I are presented the controls which substantiate the enzymatic dehydrogenation of glycerol. As can be noted from this table, there was some endogenous NADP-reducing activity; however, since both NADP and cell-free extract were present in both the reaction mixture and the blank, this activity would not be reflected in the glycerol dehydrogenase activity measured using the assay procedure. NADP was approximately 3 times as effective as coenzyme for glycerol dehydrogenase as NAD. The cell-free extract was incubated in a water bath at 80°C for 30 minutes. There was

TABLE I

COMPONENT REQUIREMENTS FOR THE ASSAY OF GLYCEROL DEHYDROGENASE

Reaction Cuvette Components	Blank Cuvette Components	Components Omitted From Blank	nmoles of NADP or NAD Reduced per Minute
CFE	H_2O	CFE	0
CFE + NADP	CFE	NADP	1.93
CFE + NADP + Glyc	CFE + NADP	Glycerol	14.5
Glycerol	H_2O	Glycerol	0
Glycerol + NADP	NADP	Glycerol	0
Glyc + NADP + CFE	NADP + CFE	Glycerol	15.0
NAD + CFE + Glyc	NAD + CFE	Glycerol	5.16

Abbreviations: CFE, cell-free extract; Glyc, glycerol; NADP, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide.

Five-tenths milliliter of 0.1 M sodium carbonate buffer, pH 11.0, was present in all cuvettes.

a complete loss of glycerol dehydrogenase activity, supporting the conclusion that the activity is enzymatic.

In order to insure the most sensitive assay and obtain the highest possible levels of end product for identification, the pH optimum for glycerol dehydrogenase was determined according to the procedure described in the preceding chapter. Figure 4 shows the pH curve determined for glycerol dehydrogenase with the pH optimum at 10.7.

The possibility of the proposed glycerol dehydrogenase being a dehydrogenase for another closely related compound was tested by determining the substrate specificity of the assay. Table II shows the results of three experiments in which extracts from three different cultures of PA-1 grown on glycerol were used. In experiment one, short chain alcohols were tested, with the highest activity being found with glycerol. The assay procedure was the same as that described in the previous chapter except that 200 µmoles of the designated substrate were added to start the reaction. With the polyhydroxy alcohols, the enzyme exhibited higher activity for D-sorbitol and D-ribitol than for glycerol, while 1,2-propanediol gave about the same level of activity as glycerol. D-mannitol was not dehydrogenated under these assay conditions. The above polyhydroxy alcohols were tried since dehydrogenases for them are listed by Barman (1969). Attempts to grow PA-1 on any of the above polyhydroxy alcohols under the same conditions used for growth on glycerol failed.

The next step in the investigation of glycerol dehydrogenase activity was to confirm Brown's observation of D-glyceraldehyde as end product. Initially radiochromatography seemed the method of choice since it is a rapid, sensitive means of detecting products of enzyme action. This technique failed to yield firm evidence of the identity of the product

Figure 4. Effect of pH on Glycerol Dehydrogenase Activity

Cultures of PA-1 were grown on glycerol in minimal medium A to an optical density at 540 nm of approximately 0.50. The cells were washed twice with saline and the resultant pellet was suspended in 5 ml of 0.02 M Tris, pH 8.5, and sonicated. The sonicate was centrifuged at 12,500 x g and the resultant cell-free extract used in glycerol dehydrogenase assays according to the procedure described in Chapter II.

The different pH values were obtained by using 0.5 M sodium carbonate buffer at various initial pH values. At the higher pH's, the pH of the reaction mixture was adjusted on a Coleman Model 39 pH meter before the addition of NADP. The pH of the reaction mixture was again checked on the pH meter upon completion of the assay.

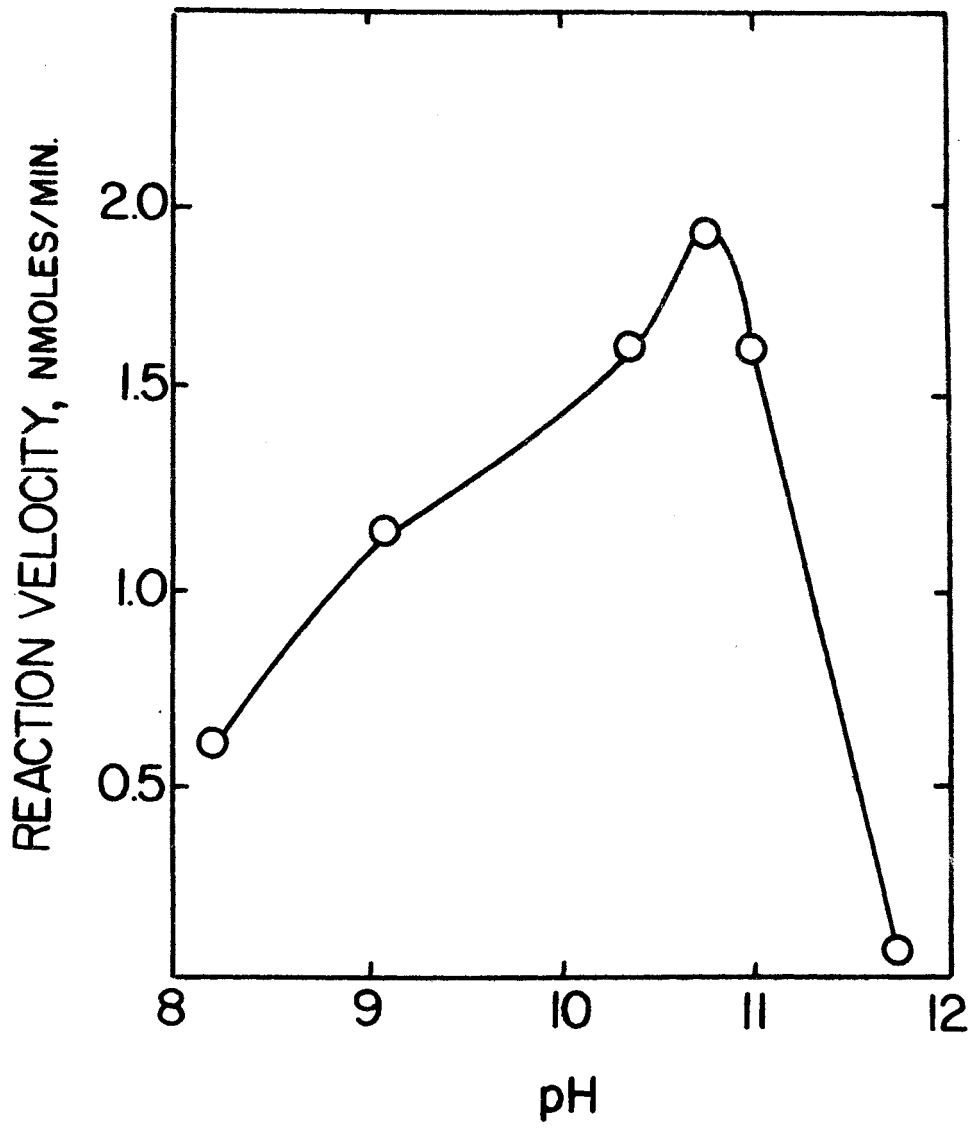

REACTION VELOCITY, NMOLES/MIN.
2.0
1.5
1.0
0.5
8
9
10
11
12
pH

TABLE II

SUBSTRATE SPECIFICITY OF GLYCEROL DEHYDROGENASE ASSAY

Substrate (200 μmoles)		Specific Activity
Expt. 1	Glycerol	4.74
	Ethanol	0.37
	Propanol	1.05
	n-Butanol	1.91
Expt. 2	Glycerol	5.17
	D-Ribitol	11.2
	D-Sorbitol	15.4
Expt. 3	Glycerol	4.34
	1,2-Propanediol	5.70

Specific activities are nmoles/min/mg protein.

because of the following problems: (1) radioactive contaminants in the ^{14}C-glycerol with the same R_f as D-glyceraldehyde in the solvent systems used, (2) high concentrations of glycerol required to give reasonably high levels of glycerol dehydrogenase activity, and (3) the apparent reactivity of the product, D-glyceraldehyde, with other components of the reaction mixture.

The first evidence for D-glyceraldehyde as the product was obtained using the 3-methyl-2-benzothiazolone hydrazone test for aliphatic aldehydes. In this experiment, 5 ml of glycerol dehydrogenase reaction mixture and 5 ml of control reaction mixture (minus the substrate, glycerol) were used to follow enzyme-produced aliphatic aldehyde and endogenously produced aliphatic aldehyde, respectively. At the same time the enzyme activity was followed on the Cary 14 dual beam recording spectrophotometer, with activity being relatively constant for the full 60 minutes. The mixtures were incubated at room temperature and samples removed from both mixtures at 0, 15, 45 and 60 minutes to be assayed for aliphatic aldehyde according to the procedure described in the preceding chapter. From each sample taken, 0.05, 0.15 and 0.50 ml aliquots were transferred to separate test tubes and brought up to 0.5 ml with distilled water. The optical densities of the various dilutions of reaction mixture with substrate were corrected for the endogenous production of aliphatic aldehyde by subtracting the optical densities of the corresponding dilutions of reaction mixture without substrate for the same incubation period and also corrected for any aliphatic aldehyde present in the reaction mixture at zero time. These optical densities were then converted to μg/ml of aliphatic aldehyde using the D-glyceraldehyde standard curve prepared simultaneously and the data are given in Table III. A control

TABLE III

3-METHYL-2-BENZOTHIAZOLONE HYDRAZONE TEST FOR ALIPHATIC ALDEHYDE PRODUCTION BY GLYCEROL DEHYDROGENASE

Reaction Mixture Incubation Time (Minutes)	µg Aliphatic[1] Aldehyde per ml Reaction Mixture
0	0
15	23.3
45	51.3
60	70.0

[1]Corrected for aliphatic aldehyde in the control at the same time as sample was removed from reaction mixture as well as for alphatic aldehyde present in the reaction mixture at zero time.

containing 0.5 ml glycerol was also tested for aliphatic aldehydes and gave negative results.

As can be seen from Table III, there was a definite increase in the level of aliphatic aldehyde during incubation of the reaction mixture containing glycerol. This does not prove that D-glyceraldehyde is the aliphatic aldehyde being produced, however. Using a combination of chromatography and the 3-methyl-2-benzothiazolone hydrazone test for aliphatic aldehyde, it was possible to obtain evidence identifying the aliphatic aldehyde as D-glyceraldehyde. A glycerol dehydrogenase reaction mixture and a reaction mixture minus glycerol were incubated for 60 minutes and 3 ml of each mixture were lyophilized. The residues were extracted with 2.5 ml volumes of absolute ethanol 4 times and the extracts for each mixture were combined and concentrated to 0.1 ml through forced evaporation to give an extract for spotting chromatograms. All of the concentrated extract for each sample was spotted on paper strips as described in Chapter II. These chromatograms were developed using descending chromatography and the solvent system, ether:acetic acid:water (15:3:1,v:v:v). The chromatograms were air-dried and 5 ml of 0.4 per cent aqueous 3-methyl-2-benzothiazolone hydrazone were pipetted on to each strip, which was then placed in a 100°C oven for 3 minutes followed by air drying. Under a long wave UV light, a fluorescent spot was observed for the extract from the glycerol dehydrogenase reaction mixture, which had the same R_f as D-glyceraldehyde on a control chromatogram detected by Gordon's periodic acid-benzidine system, while the extract from the reaction mixture with no substrate gave no visible spots.

Another more conclusive experiment was conducted using semicarbazide hydrochloride as an aldehyde trapping agent to confirm the conclusion

reached with the 3-methyl-2-benzothiazolone hydrazone experiments. Buswell and Mahmood (1972) reported a 65 per cent recovery of theoretical formaldehyde production in a reaction mixture containing cell-free extract when semicarbazide hydrochloride was used to trap formaldehyde whereas no formaldehyde was detectable in the absence of trapping agent. It should also be noted that use of a trapping agent would prevent the loss of D-glyceraldehyde due to its interaction with other reaction mixture components, which was noted earlier. Since glycerol dehydrogenase has a broad pH range, it was possible to conduct this experiment at pH 7.8, a pH at which semicarbazones are rapidly formed (Shriner et al., 1962). The pH of 7.8 for the reaction mixture was attained by replacing the 0.1 M sodium carbonate buffer, pH 11.0, normally used, with 0.2 M sodium phosphate buffer, pH 8.0. When 0.1 ml of neutralized 0.2 N semicarbazide hydrochloride was added to 1 ml of glycerol dehydrogenase reaction mixture at pH 7.8, there was no apparent change in glycerol dehydrogenase activity. The activity of glycerol dehydrogenase in the presence of neutral semicarbazide was highest during the first ten minutes of incubation with a gradual decrease in activity over the next 40 minutes. The procedure used for semicarbazide trapping of D-glyceraldehyde, and preparation and detection of semicarbazide derivatives was presented in Chapter II.

Two 3 ml glycerol dehydrogenase reaction mixtures were incubated for 30 minutes at room temperature in the presence of semicarbazide hydrochloride with one containing 0.3 ml of 80 per cent glycerol and the other 0.3 ml of a 159 nanomolar solution of glycerol-U-^{14}C (14.3 mCi/m mole) as substrate. The semicarbazones were prepared as previously described and the spots obtained by chromatography are shown in Figure 5.

Figure 5. Silica Gel Thin-layer Chromatogram of Semicarbazones of Glycerol Dehydrogenase Product, Controls and Standards

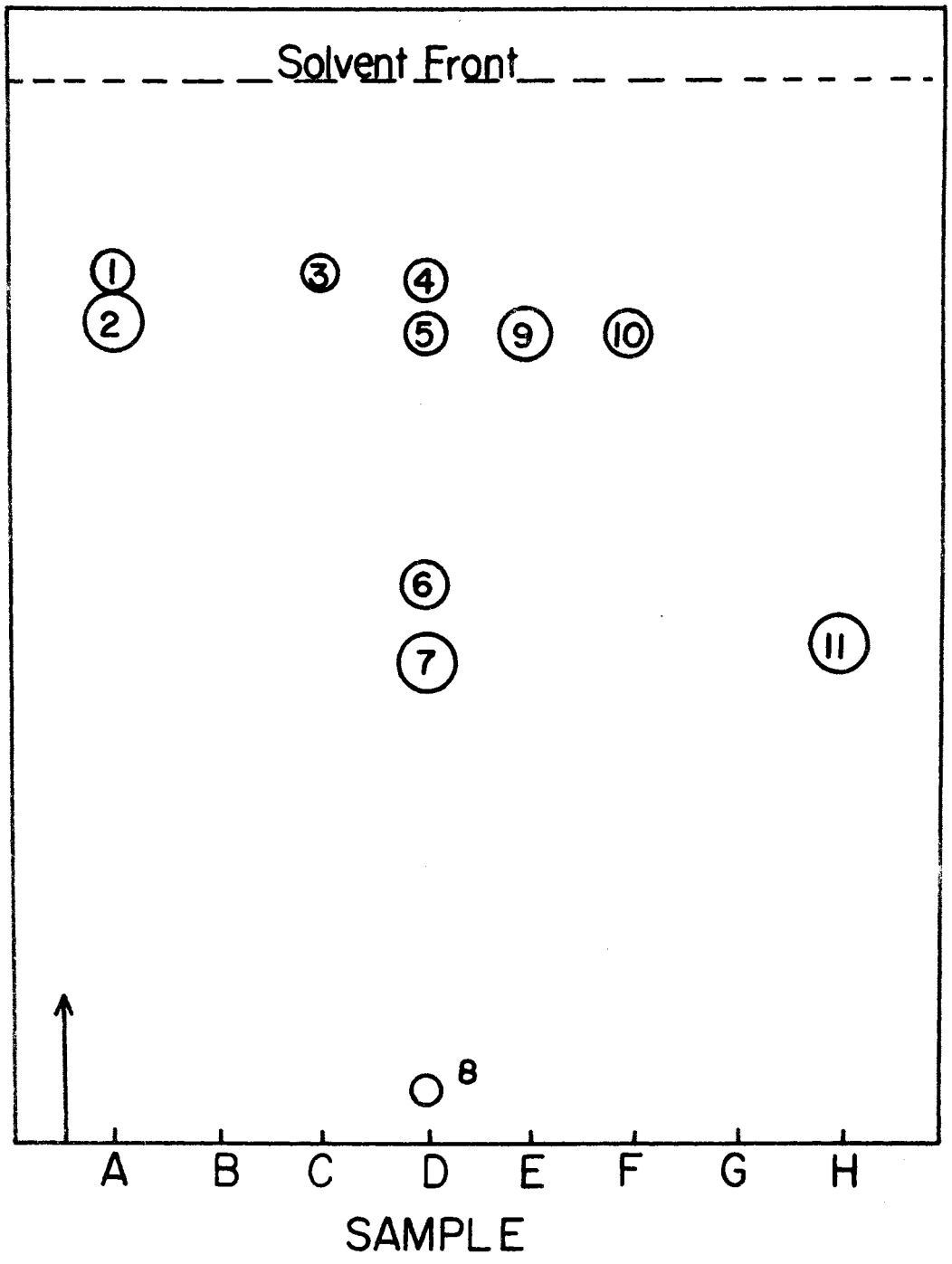
The semicarbazones for each sample were obtained as indicated in the text of this chapter and the preceding one. The chromatogram was developed using the solvent system: t-amyl alcohol:aqueous ammonia (sp. gr. 0.90): n-propanol (65:35:5, v:v:v). Semicarbazones were detected by spraying with 0.4 per cent 2,4-dinitrophenylhydrazone in 2 N HCl.

Samples used to prepare semicarbazones:

A. Complete reaction mixture after 30 min. incubation.
B. Complete reaction mixture at zero time.
C. Reaction mixture, glycerol omitted, 30 min. incubation.
D. Reaction mixture, glycerol and NADP omitted, D-glyceraldehyde added, 30 min. incubation.
E. D-glyceraldehyde and water, 30 min. incubation.
F. D-glyceraldehyde.
G. Glycerol.
H. Dihydroxyacetone.

The color and color intensity (light, +; moderate, ++; dark, +++) for each numbered spot on the chromatogram is as follows:

1. yellow (+).
2. yellow-orange (++).
3. yellow (+).
4. grey (++).
5. yellow (+).
6. brown (+).
7. orange (+++).
8. brown (++).
9. yellow-orange (++).
10. yellow-orange (++).
11. orange (+++).


Solvent Front
1
2
3
4
5
9
10
6
7
11
8
A
B
C
D
E
F
G
H
SAMPLE

The first reaction mixture (Figure 5, A) yielded a major spot (number 2) with an R_f of 0.77, which is the same as that for the standard D-glyceraldehyde semicarbazone (spot number 10), and a minor spot (number 1) with an R_f of 0.82. When the thin-layer chromatogram of the second reaction mixture using radioactive glycerol as substrate was scanned on a Packard Radiochromatogram Scanner Model 7201, there were two radioactive semicarbazone peaks. Approximately 80 per cent of the radioactivity was found in a semicarbazone peak with an R_f of 0.77; the remaining 20 per cent of the radioactivity was found in a semicarbazone peak with an R_f of 0.39. The position of the first peak again matched the R_f of the D-glyceraldehyde semicarbazone (spot number 10) while the other peak, R_f 0.39, was relatively close to the position of the standard dihydroxyacetone semicarbazone (spot number 11) which had an R_f of 0.45. The minor radioactive peak might be explained by the isomerization of D-glyceraldehyde to dihydroxyacetone under the alkaline conditions of the reaction mixture (Gutsche et al., 1967). Since there was no radioactive semicarbazone with an R_f of 0.82 corresponding to the spot (number 1) observed in the first reaction mixture, this spot must represent a semicarbazone formed with some other component of the reaction mixture.

Four controls were run simultaneously with the two reaction mixtures given above and are also shown in Figure 5. The first control was designed to test for the presence of compounds capable of forming semicarbazones at the start of the reaction and also to test for any generation of compounds capable of forming semicarbazones in the presence of glycerol during the 10 minute, 100°C deproteinization step. This control, consisting of the reaction mixture plus 80 per cent glycerol as substrate, with no incubation at room temperature and immediate deproteini-

zation, gave no semicarbazone spots (Figure 5, B). There should have been a semicarbazone spot with an R_f of 0.82 if this spot (number 1) had represented a semicarbazone formed with some other component initially present in the reaction mixture or formed during deproteinization. Semicarbazone formation with components of the reaction mixture incubated without glycerol is represented in the second control (Figure 5, C). Since the semicarbazone spot (number 3) with an R_f of 0.82 was present in this control, it can be concluded that this spot represents a compound formed endogenously during incubation.

The third control (Figure 5, D) serves to indicate whether D-glyceraldehyde added to the reaction mixture can interact with semicarbazide to form semicarbazones which are detectable with this experimental procedure. NADP was omitted from the reaction mixture since glyceraldehyde dehydrogenase might function under the conditions of this assay and remove added glyceraldehyde. This control, containing 0.4 ml of 0.1 M D-glyceraldehyde in place of the NADP and glycerol normally present in the reaction mixture, gave semicarbazones with R_f's of 0.05 (spot number 8), 0.45 (spot number 7), 0.52 (spot number 6), 0.77 (spot number 5) and 0.82 (spot number 4). Interaction of D-glyceraldehyde with other components of the reaction mixture, before the D-glyceraldehyde could be trapped by semicarbazide, could explain the new semicarbazone spots (numbers 8 and 6) with R_f's of 0.05 and 0.52. Gutsche et al. (1967) found that in alkaline solutions D-glyceraldehyde formed condensation products including D-fructose, D-sorbose and DL-dendroketose. The semicarbazone spot (number 7) with an R_f matching that of dihydroxyacetone semicarbazone (R_f of 0.45, spot number 11) was much more intense than the spot (number 5) with an R_f matching that of D-glyceraldehyde semi-

carbazone (spot number 10). Rapid isomerization of D-glyceraldehyde to dihydroxyacetone (prior to reaction with semicarbazide), which is favored under the alkaline conditions of this assay (Gutsche et al., 1967), would explain the more intense dihydroxyacetone semicarbazone spot (number 7). The fourth control (Figure 5, E) with 0.4 ml of 0.1 M D-glyceraldehyde in 2.6 ml of water gave only one semicarbazone spot (number 9) with an R_f of 0.77 matching that of the D-glyceraldehyde semicarbazone (spot number 10) which shows that little isomerization took place in the acid pH of the water. Glycerol (Figure 5, G) gave no detectable semicarbazones.

Since a semicarbazone with an R_f of 0.77 appeared in all samples containing D-glyceraldehyde, including the glycerol dehydrogenase reaction mixture incubated for 30 minutes and the D-glyceraldehyde standard, it can be concluded that D-glyceraldehyde is the product of the action of glycerol dehydrogenase on glycerol. This would lend credence to the results obtained with 3-methyl-2-benzothiazolone hydrazone as well.

If D-glyceraldehyde is the product formed by glycerol dehydrogenase, it should be possible to measure the reverse activity, i.e., to show oxidation of $NADPH_2$ in the presence of D-glyceraldehyde and cell-free extract containing glycerol dehydrogenase. Accordingly an assay for this activity was developed. The assay mixture included: 0.7 ml of 0.2 M sodium phosphate buffer, pH 6.0; 0.1 ml of 0.001 M $NADPH_2$; 0.1 ml of 0.1 M D-glyceraldehyde; and 0.1 ml of cell-free extract. Controls for the assay are given in experiment 1, Table IV. Since endogenous $NADPH_2$-oxidizing activity was noted under these assay conditions, both $NADPH_2$ and cell-free extract were added to the reaction mixture and the blank, and D-glyceraldehyde was added to the experimental cuvette to

TABLE IV

COMPONENT REQUIREMENTS FOR THE ASSAY OF REDUCTION OF D-GLYCERALDEHYDE BY GLYCEROL DEHYDROGENASE

Reaction Cuvette Components	Blank Cuvette Components	Components Omitted From Blank	nmoles of NADP or NAD Reduced per Minute
Expt. 1			
$NADPH_2$	H_2O	$NADPH_2$	0
$NADPH_2$ + GAld	$NADPH_2$	GAld	0
$NADPH_2$ + GAld + CFE	$NADPH_2$ + CFE	GAld	101
$NADPH_2$ + GAld + CFE + EtSH	$NADPH_2$ + CFE + EtSH	GAld	35.5
GAld	H_2O	GAld	0
$NADPH_2$ + CFE	CFE	$NADPH_2$	5.32
Exp. 2			
$NADPH_2$ + GAld + CFE	$NADPH_2$ + CFE	GAld	306
$NADH_2$ + GAld + CFE	$NADH_2$ + CFE	GAld	59.7

Abbreviations: CFE, cell-free extract; GAld, D-glyceraldehyde; $NADH_2$, reduced nicotinamide adenine dinucleotide; EtSH, mercaptoethanol; $NADPH_2$, reduced nicotinamide adenine dinucleotide phosphate.

Seven-tenths milliliter of 0.2 M sodium phosphate buffer, pH 6.0, was present in all cuvettes.

start the assay. No interaction between D-glyceraldehyde and individual reaction mixture components was observed at this pH. Mercaptoethanol decreased activity. The coenzyme, $NADPH_2$, was slightly more than 5 times more effective than $NADH_2$ as shown in experiment 2, Table IV. The assay procedure was not optimized; however, at pH 6.0 enzyme activity was much higher than at pH 7.0 or 7.5 which agrees with the pH optimum (less than 7.0) of a similar enzyme from liver studied by Moore (1959). The enzyme was inactivated by incubation in an $80^{o}C$ water bath for 30 minutes.

The substrate specificity of the glycerol dehydrogenase assay in the reverse direction is shown in Table V. Of the short chain aldehydes, D-glyceraldehyde served as the best substrate. The relatively high level of enzyme activity with benzaldehyde is unexpected; possibly another enzyme may be utilizing it as substrate under the conditions of this assay. No activity was found with dihydroxyacetone as substrate.

The specific activity of glycerol dehydrogenase with D-glyceraldehyde as substrate was approximately 100 times the specific activity in the reverse direction, i.e., formation of D-glyceraldehyde, when the same cell-free extract was used. The cell-free extract was diluted 1 to 10 with 0.02 M Tris buffer, pH 8.5, to allow measurement of $NADPH_2$ oxidation in the presence of D-glyceraldehyde.

The product of the enzymatic reduction of D-glyceraldehyde was identified as glycerol. This was accomplished by adding 4 μcuries of labelled D-glyceraldehyde to 3 ml of a modified reaction mixture for glycerol dehydrogenase reduction of D-glyceraldehyde followed by incubation at room temperature for 15 minutes. The modification involved use of undiluted cell-free extract instead of the standard 1 in 10 diluted

TABLE V

SUBSTRATE SPECIFICITY OF ASSAY FOR GLYCEROL DEHYDROGENASE REDUCTION OF ALDEHYDES

Substrate (10 μmoles)	Specific Activity
D-Glyceraldehyde	355
Acetaldehyde	0
Glyoxal	110
Propionaldehyde	43.5
Butyraldehyde	55.3
Isobutyraldehyde	14.2
Benzaldehyde	186

Specific activities are nmole/min/mg protein.

cell-free extract, and addition of 0.02 M $NADPH_2$ instead of the standard 0.001 M normally added for enzyme assay. Both of these modifications were designed to increase the amount of product produced by glycerol dehydrogenase. Four reaction mixture controls were incubated zero minutes at room temperature and varied from the reaction mixture above only by the deletion of $NADPH_2$ and the type of substrate present. The substrates present in each of the controls included: (1) 4 μcuries labelled D-glyceraldehyde, (2) 4 μcuries labelled glycerol, (3) 0.09 mmoles unlabelled D-glyceraldehyde, and (4) 0.09 mmoles unlabelled glycerol. $NADPH_2$ was omitted from all controls due to the high reducing activity of the glycerol dehydrogenase in the cell-free extract. All reaction mixtures were quick-frozen in a dry ice-acetone bath and lyophilized. The dry samples were resuspended in 2 ml of absolute ethanol and centrifuged. Ethanol was used to extract since glycerol is soluble in ethanol and D-glyceraldehyde is slightly soluble in ethanol. This allowed separation of the labelled compounds from most of the other components of the reaction mixture. It was shown that the amounts of labelled D-glyceraldehyde used in this experiment were readily extracted by ethanol.

The supernatant from each sample was carefully removed with a Pasteur pipet and concentrated under a gentle stream of air to approximately 0.1 ml. All of the concentrated ethanol extract for each sample was spotted for chromatography as described in Chapter II except that for controls 3 and 4 only 50 μl were spotted. In addition, 0.5 μcuries of labelled D-glyceraldehyde and glycerol were spotted for chromatography.

All chromatograms were developed using the solvent system ether: acetic acid:water, 13:3:1. This solvent system gave excellent separation of D-glyceraldehyde and glycerol, which had R_f's of 0.23 and 0.58, re-

spectively. Upon scanning the chromatogram of the 15 minute reaction mixture, it was found that the major portion of the labelled D-glyceraldehyde had been converted to glycerol since there was a very prominent radioactive peak with an R_f which matched the R_f of glycerol. In addition there was another minor, faster moving radioactive peak with an R_f of 0.74 which remains unidentified. The extracts of the labelled and unlabelled reaction mixture controls produced spots on chromatograms which matched the R_f's of D-glyceraldehyde and glycerol. It should be emphasized that there was no major radioactive peak for labelled D-glyceraldehyde from the 15 minute reaction mixture, indicating almost complete conversion to glycerol.

Aldehyde Dehydrogenase

Oxidation of the D-glyceraldehyde produced by the action of glycerol dehydrogenase on glycerol was considered to be the next logical step in a nonphosphorylated pathway for glycerol metabolism. In earlier investigations, Ding (1972) had shown that P. aeruginosa strain PA-1 lacked D-glyceraldehyde kinase which was confirmed by the assay procedures of Sillero et al. (1969) during this investigation. Cell-free extracts of PA-1 grown in minimal medium containing glycerol as the sole carbon source were found to contain an aldehyde dehydrogenase capable of oxidizing D-glyceraldehyde as measured by the assay procedure described for glycerol-grown cells in Chapter II. Tigerstrom and Razzell (1968) had previously found an aldehyde dehydrogenase in cell-free extracts of P. aeruginosa grown with ethanol as the sole carbon source. In both instances the enzymes were able to utilize D-glyceraldehyde as a substrate. In the present study experiments were conducted so a comparison of the

aldehyde dehydrogenases produced in the presence of the two different carbon sources could be made. The comparison of properties would indicate whether they were the same aldehyde dehydrogenase or two different ones.

The assay procedure for aldehyde dehydrogenase from ethanol-grown cells was described in the preceding chapter. Tigerstrom and Razzell (1968) conducted their assay at pH 7.2, which was far below the pH optimum of 8.0 to 8.6, because they obtained a linear reaction rate for a longer period of time at that pH. Since the level of aldehyde dehydrogenase in glycerol-grown cells was quite low, it was essential to develop an assay at a pH closer to the optimum. The assay method developed for glycerol-grown cells was also described in Chapter II. The controls for this assay procedure are shown in Table VI. Endogenous NAD-reducing activity was shown to be present, and this was compensated for by using cell-free extract and NAD in both the reaction mixture and the blank. Since there was an absolute requirement for a reducing agent for aldehyde dehydrogenase activity, mercaptoethanol was also included in both the reaction mixture and blank. The inclusion of mercaptoethanol in both the reaction mixture and blank compensated for its enhancement of endogenous NAD-reducing activity. Although D-glyceraldehyde reduced NAD chemically to a minor extent, as has been reported by Needham et al. (1951), D-glyceraldehyde was chosen as the component to be omitted from the blank and added to the reaction mixture to initiate the assay for aldehyde dehydrogenase. D-Glyceraldehyde reduction of NAD resulted in the smallest error in the aldehyde dehydrogenase assay and this was easily corrected for when necessary. Potassium ions, 0.1 M in the reaction mixture, had essentially no effect on aldehyde dehydrogenase activ-

TABLE VI

COMPONENT REQUIREMENTS FOR THE ASSAY OF ALDEHYDE DEHYDROGENASE

Reaction Cuvette Components	Blank Cuvette Components	Component Omitted From Blank	nmoles of NAD or NADP Reduced per Minute
CFE	H_2O	CFE	0
NAD + CFE	NAD	CFE	0.81
EtSH + CFE	EtSH	CFE	0
EtSH + NAD + CFE	EtSH + NAD	CFE	2.74
GAld	H_2O	GAld	0
GAld + CFE	GAld	CFE	0
GAld + NAD + CFE	GAld + NAD	CFE	0
GAld + NAD	NAD	GAld	0.16
EtSH + NAD + CFE + GAld	EtSH + NAD + CFE	GAld	2.74
EtSH + NAD + CFE + GAld + KCl	EtSH + NAD + CFE + KCl	GAld	2.90
EtSH + NADP + CFE + GAld	EtSH + NADP + CFE	GAld	0.81

Abbreviations: CFE, cell-free extract; GAld, D-glyceraldehyde; EtSH, mercaptoethanol; NADP, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide.

Four-tenths of a milliliter of 0.1 M Tris buffer, pH 9.1, was present in each cuvette.

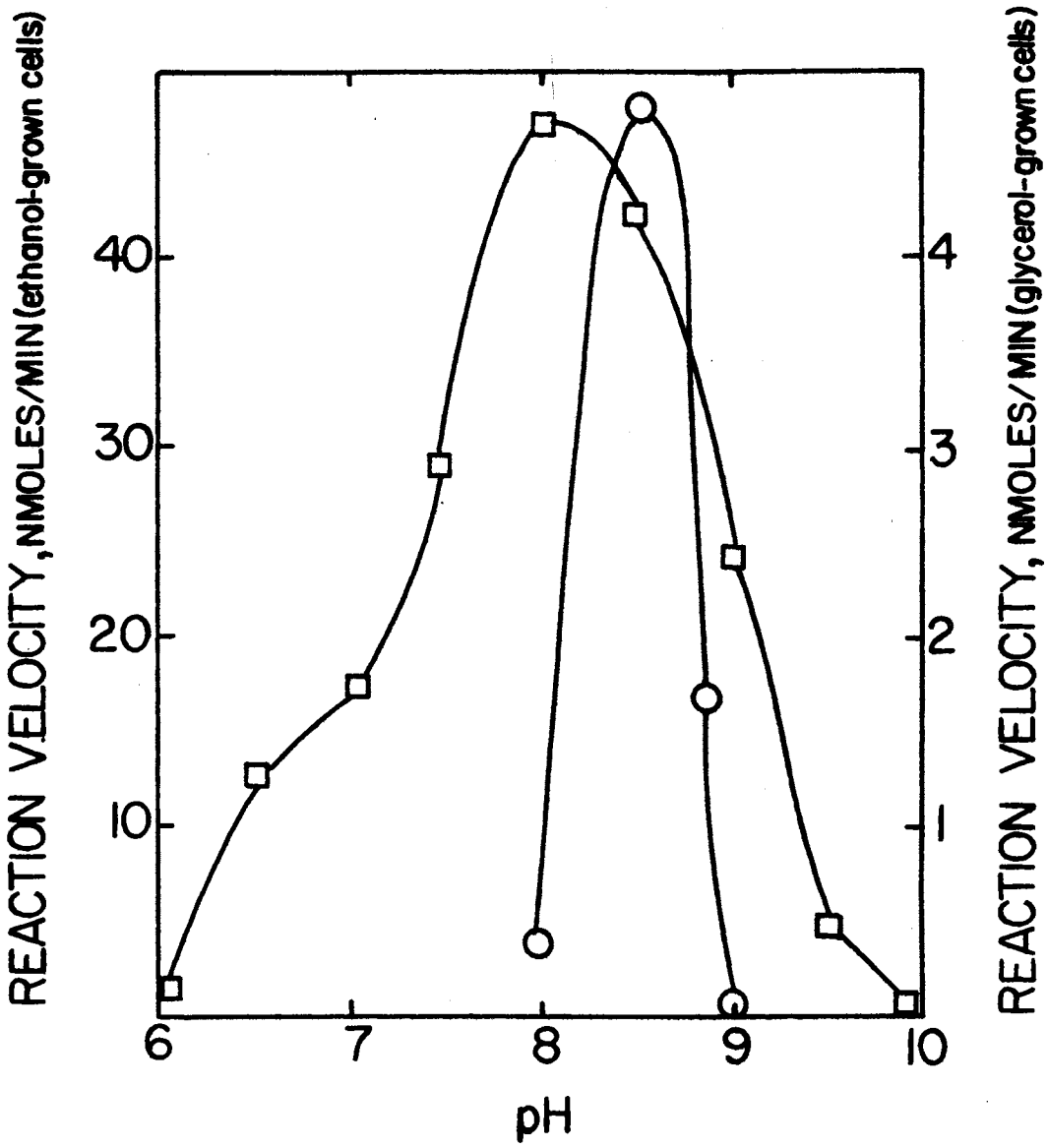
ity from glycerol-grown cells (Table VI). Tigerstrom and Razzell (1968) found that the activity of their aldehyde dehydrogenase was increased 12.5-fold by addition of potassium ion. Since the potassium ion enhancement of aldehyde dehydrogenase was not significant for glycerol-grown cell extracts, potassium chloride was not generally included in assays for this enzyme. As coenzyme, NAD was almost 4 times as effective with the enzyme as NADP. The aldehyde dehydrogenase activity was demonstrated to be enzymatic in nature since heating the cell-free extract in a water bath at 80°C for 30 minutes resulted in complete loss of activity.

The pH curves for the aldehyde dehydrogenases produced in cells grown on the two different carbon sources are presented in Figure 6. Since the pH optima for these aldehyde dehydrogenases were at alkaline pH's, a modification of the assay procedure for both enzymes was necessary. Cell-free extract was added to the reaction mixture to initiate the assay in both cases instead of D-glyceraldehyde. The reason for the above modification was the reaction of NAD with D-glyceraldehyde in the presence of hydroxyl ions to yield a product which had an absorption maximum at 340 nm (Burton and Kaplan, 1954) coinciding with the absorption maximum of $NADH_2$. The more alkaline the pH, the higher the rate at which this product is formed. Inclusion of D-glyceraldehyde and NAD in both the reaction mixture and the blank compensated for formation of the interfering product. The endogenous NAD-reducing activity in the cell-free extract was measured separately and corrected for when significant. Figure 6 shows that the aldehyde dehydrogenase from glycerol-grown cells had a very sharp pH optimum near pH 8.5, while the pH optimum of the aldehyde dehydrogenase from ethanol-grown cells was near pH 8.0. The aldehyde dehydrogenase activity from glycerol-grown cells was approxi-

Figure 6. Effect of pH on Aldehyde Dehydrogenase Activity

Cultures of PA-1 were grown in 0.5 per cent ethanol or 0.5 per cent glycerol in minimal medium A to an optical density of approximately 0.50, harvested and washed twice with saline. The cell pellet was suspended in 5 ml of 0.02 M Tris, pH 8.5, sonicated, and centrifuged at 12,500 x g. The cell-free extract was used in the aldehyde dehydrogenase assays described in Chapter II.

The aldehyde dehydrogenase activity of ethanol-grown cells is represented by the open squares (□), while the aldehyde activity of glycerol-grown cells is represented by the open circles (○).


REACTION VELOCITY, NMOLES/MIN (ethanol-grown cells)
REACTION VELOCITY, NMOLES/MIN (glycerol-grown cells)
40
30
20
10
4
3
2
1
6
7
8
9
10
pH

mately one-tenth of that from ethanol-grown cells.

The substrate specificities of the aldehyde dehydrogenases from glycerol- and ethanol-grown cells were examined and the results are presented in Table VII. Two different concentrations of each aldehyde were used to examine aldehyde dehydrogenase activity from glycerol-grown cells to determine whether substrate inhibition occurred at the higher concentrations. Tigerstrom and Razzell (1968) reported for the aldehyde dehydrogenase from ethanol-grown cells that activity decreased as substrate concentration was increased. As Table VII shows, a similar effect with increased substrate was found for the enzyme from glycerol-grown cells only with isobutyraldehyde, cinnamaldehyde and benzaldehyde, and these differences are quite small. The aldehyde dehydrogenase from glycerol-grown cells was most active when propionaldehyde and n-butyraldehyde were the substrates, with acetaldehyde, glyoxal, D-glyceraldehyde and isobutyraldehyde also giving high levels of activity. The aromatic aldehydes served as relatively poor substrates. When PA-1 was grown on ethanol, aldehyde dehydrogenase had the highest activity with isobutyraldehyde as substrate. Propionaldehyde was utilized at about one-half the rate of isobutyraldehyde and D-glyceraldehyde at about 35 per cent. Cinnamaldehyde, acetaldehyde, and glyoxal were poor substrates. The substrate specificity of aldehyde dehydrogenase from ethanol-grown PA-1 does not agree with the relative activities of the aldehyde dehydrogenase studied by Tigerstrom and Razzell (1968). Also, it is readily apparent that the substrate specificities of the aldehyde dehydrogenases from ethanol- and glycerol-grown PA-1 differ. These have been calculated as ratios of activities with each substrate compared to the activity with D-glyceraldehyde as substrate and these are shown in Table VII.

TABLE VII

SUBSTRATE SPECIFICITY OF ALDEHYDE DEHYDROGENASE ASSAYS

Substrate	Glycerol-Grown Cells[1]			Ethanol-Grown Cells	
	1 µmole	30 µmoles		30 µmoles	
	Specific Activity	Specific Activity	Activity Compared to D-glyceraldehyde	Specific Activity	Activity Compared to D-glyceraldehyde
Acetaldehyde	0.47	1.55	1.05	2.69	0.24
Glyoxal	0.54	1.28	0.86	4.30	0.39
D-glyceraldehyde	0.54	1.48	--	11.1	--
Propionaldehyde	1.55	2.02	1.36	12.2	1.55
n-Butyraldehyde	1.34	1.95	1.32	9.35	0.84
Isobutyraldehyde	1.81	1.55	1.05	30.6	2.77
Cinnamaldehyde	0.27	0.20	0.13	0.64	0.06
Benzaldehyde	0.07	0.20	0.13	8.60	0.78

[1]Aldehyde dehydrogenase activity from glycerol-grown cells was determined at pH 8.5 according to the procedure in Chapter II while in ethanol-grown cells it was determined according to the procedure of Tigerstrom and Razzell (1968).

Specific activities are nmoles/min/mg protein.

Heat inactivation of the aldehyde dehydrogenases produced by cells grown on the two different carbon sources was used to determine whether the aldehyde dehydrogenases were actually the same enzyme. Cell-free extract, or diluted cell-free extract, with aldehyde dehydrogenase activity was placed in a prewarmed tube in a 60°C water bath. At the end of 30 minutes incubation, the cell-free extracts were examined for residual aldehyde dehydrogenase activity. The cell-free extract of glycerol-grown cells retained at least 75 per cent of its initial aldehyde dehydrogenase activity as compared with less than 50 per cent activity remaining in the cell-free extract from ethanol-grown cells. The aldehyde dehydrogenase from glycerol-grown cells is much more heat stable than is the aldehyde dehydrogenase from ethanol-grown cells.

If glyceric acid is the product formed by aldehyde dehydrogenase in the oxidation of D-glyceraldehyde, it should be possible to measure aldehyde dehydrogenase activity in the reverse direction by observing the oxidation of $NADH_2$ in the presence of glyceric acid and cell-free extract containing aldehyde dehydrogenase. It was possible to detect such enzyme activity using the following assay procedure. The assay mixture contained: 0.7 ml of 0.5 M sodium carbonate buffer, pH 9.0; 0.1 ml of 0.001 M $NADH_2$; 0.1 ml of 5 per cent glyceric acid; and 0.1 ml of cell-free extract. Controls for this assay are given in Table VIII. Endogenous $NADH_2$ oxidase activity at alkaline pH's had been observed at the beginning of this investigation; therefore, $NADH_2$ and cell-free extract were included in both the reaction mixture and the blank. Table VIII shows that there is no oxidation of $NADH_2$ by glyceric acid in the absence of cell-free extract and no interaction between glyceric acid and cell-free extract to produce a product which absorbs light at 340 nm.

TABLE VIII

COMPONENT REQUIREMENTS FOR MEASUREMENT OF THE REDUCTION OF GLYCERIC ACID BY ALDEHYDE DEHYDROGENASE

Reaction Cuvette Components	Blank Cuvette Components	Component Omitted From Blank	nmoles of $NADPH_2$ or $NADH_2$ Oxidized/min
Expt. 1			
$NADH_2$ + GA	$NADH_2$	GA	0
CFE + GA	CFE	GA	0
CFE + $NADH_2$ + GA	CFE + $NADH_2$	GA	3.71
Expt. 2			
No Preincubation[1]			
CFE + $NADH_2$ + GA	CFE + $NADH_2$	GA	2.58
Preincubation[2]			
CFE + $NADH_2$ + GA	CFE + $NADH_2$	GA	4.84
Preincubation			
CFE + $NADPH_2$ + GA	CFE + $NADPH_2$	GA	48.4

Abbreviations: CFE, cell-free extract; GA, glyceric acid; $NADH_2$, reduced nicotinamide adenine dinucleotide; $NADPH_2$, reduced nicotinamide adenine dinucleotide phosphate.

[1]Glyceric acid was added to $NADH_2$ and cell-free extract in buffer to start assay.

[2]Glyceric acid was added to $NADH_2$ or $NADPH_2$ in buffer and incubated 1 minute at room temperature prior to addition of cell-free extract.

Seven-tenths of a milliliter of 0.5 M sodium carbonate buffer, pH 9.0, was present in each cuvette.

It should be noted from experiment two that preincubation of glyceric acid with $NADH_2$ prior to addition of cell-free extract resulted in a higher level of enzyme activity. For this reason, glyceric acid was added to the reaction mixture (and an equal amount of water to the blank) followed by incubation at room temperature for 1 minute prior to the addition of cell-free extract to both the reaction mixture and blank. Late in this investigation when $NADPH_2$ became available in this laboratory, it was found that $NADPH_2$ was oxidized 10 times as rapidly as $NADH_2$ in the presence of glyceric acid under the above assay conditions as shown in experiment two.

Aldehyde dehydrogenase-catalyzed reduction of glyceric acid using cell-free extract from glycerol-grown cells was approximately 50 per cent faster than the oxidation of D-glyceraldehyde. The ethanol-grown cells exhibited very high levels of aldehyde dehydrogenase activity resulting in the oxidation of D-glyceraldehyde, but no oxidation of either $NADH_2$ or $NADPH_2$ in the presence of glyceric acid was observed under the conditions of the above assay.

The substrate specificity of the reduction by aldehyde dehydrogenase from glycerol-grown cells was examined in part to determine whether it would lend support to glyceric acid as the end product of aldehyde dehydrogenase oxidation of D-glyceraldehyde. The results of this study are presented in Table IX. High aldehyde dehydrogenase reducing activity was shown only when glyceric acid was present and only low levels of activity were present when β-hydroxybutyrate and pyruvate were substrates.

D-Glycerate has been identified, at least tentatively, as the product of the enzymatic oxidation of D-glyceraldehyde by extracts

TABLE IX

SUBSTRATE SPECIFICITY OF REDUCTION OF ORGANIC ACIDS BY ALDEHYDE DEHYDROGENASE

Substrate (50 μmoles)	Specific Activity
Glyceric acid	6.00
Sodium glyoxylate	0
Sodium lactate	0
Sodium butyrate	0
β-Hydroxybutyrate	1.03
Sodium pyruvate	0.51

Specific activities are nmole/min/mg protein.

from glycerol-grown cells. This was accomplished using approximately 4 μcuries of purified D-glyceraldehyde-U-^{14}C in 3 ml of the aldehyde dehydrogenase reaction mixture at pH 8.5. The reaction mixture was incubated at room temperature for 1 hour and immediately quick-frozen in a dry ice-acetone bath and lyophilized. Controls which were incubated for zero time and lyophilized included: (1) 4 μcuries of purified D-glyceraldehyde-U-^{14}C in the reaction mixture, (2) 4 μcuries of D-glycerate-U-^{14}C in the reaction mixture, (3) 0.09 mmoles of unlabelled D-glyceraldehyde in the reaction mixture, and (4) 0.09 mmoles of unlabelled D-glyceric acid in the reaction mixture. The resultant dry samples were resuspended in 0.2 ml of distilled water followed by centrifugation for 10 minutes at 12,500 x g at room temperature. One hundred microliters of the supernatant from each sample were spotted for paper chromatography as described in Chapter II. At the same time 0.5 μcuries each of D-glyceraldehyde-U-^{14}C and D-glycerate-U-^{14}C were also spotted on separate strips of chromatography paper. The chromatography strips were developed using the solvent system: ether:acetic acid:water, 13:3:1. This solvent system gave good separation of D-glyceraldehyde and D-glycerate, which had R_f's of 0.23 and 0.48, respectively. D-Glyceraldehyde gave another minor spot with an R_f of 0.40. The chromatography strips containing radioisotopes were scanned and the unlabelled controls were treated according to the procedures described in Chapter II.

The controls containing the unlabelled D-glyceraldehyde and D-glycerate in the reaction mixture gave R_f's which matched the R_f's of the radioisotope controls for the two compounds. The chromatogram from the reaction mixture incubated for one hour gave a minor radioactive peak with an R_f of 0.48 which matches the R_f of the D-glycerate-U-^{14}C control.

The radioactivity of this D-glycerate peak from the reaction mixture was relatively low, as would be expected from the low level of aldehyde dehydrogenase activity in the extract.

Glycerate Dehydrogenase

If the product of aldehyde dehydrogenase action on D-glyceraldehyde is glyceric acid, the next enzyme in the nonphosphorylated pathway for glycerol metabolism would logically involve either a glycerate kinase or a glycerate dehydrogenase. No glycerate kinase was detectable in the cell-free extracts of PA-1 grown on glycerol or glyceric acid. The procedures of Kornberg and Gotto (1961) and Kohn and Jakoby (1966) were used for testing for glycerate kinase.

A glycerate dehydrogenase was observed to be present when the following assay procedure was used. The reaction mixture included: 0.7 ml of 0.5 M sodium carbonate buffer, pH 10.5; 0.1 ml of 5 per cent glyceric acid; 0.1 ml of 0.02 M NADP; and 0.1 ml of cell-free extract.

The controls for the above assay procedure are presented in Table X. Although significant endogenous NADP-reducing activity was shown to be present in the cell-free extract, it was necessary to include both NADP and glyceric acid in the reaction mixture and the blank because of the reduction of NADP by glyceric acid in the absence of cell-free extract. Addition of NADP to the reaction mixture was used to start the glycerate dehydrogenase assay. It was necessary to determine separately the endogenous NADP-reducing activity present in the cell-free extract and correct for it when it had a significant effect on experimental results. In experiment 2, it is shown that NADP is a slightly more effective coenzyme than NAD. The enzymatic nature of the glyceric acid oxi-

TABLE X

COMPONENT REQUIREMENTS FOR THE ASSAY OF GLYCERATE DEHYDROGENASE

Reaction Cuvette Components	Blank Cuvette Components	Component Omitted From Blank	nmoles of NAD or NADP Reduced per Minute
Expt. 1			
CFE	H_2O	CFE	0
CFE + NADP	CFE	NADP	0.80
CFE + NADP + GA	CFE + GA	NADP	5.97
GA	H_2O	GA	0
GA + CFE	GA	CFE	0
NADP + GA	NADP	GA	1.13
Expt. 2			
GA + NADP + CFE	GA + NADP	CFE	6.45
GA + NAD + CFE	GA + NAD	CFE	5.16

Abbreviations: CFE, cell-free extract; GA, glyceric acid; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

Seven-tenths of a milliliter of 0.5 M sodium carbonate buffer, pH 10.5, was present in each cuvette.

dation was shown by the complete loss of activity following incubation of cell-free extract for 30 minutes in an 80°C water bath.

The pH optimum of 10.2 for glycerate dehydrogenase is shown in Figure 7. The pH optimum was determined according to the procedure described in Chapter II. By using 0.7 ml of 0.5 M sodium carbonate buffer, pH 10.5, in the reaction mixture, the pH optimum of 10.2 for glycerate dehydrogenase was attained.

The results of the examination of the substrate specificity of glycerate dehydrogenase are shown in Table XI. The enzyme was shown to be specific for glyceric acid with much lower activity exhibited in the presence of glyoxylate, lactate, butyrate and β-hydroxybutyrate. No NADP-reducing activity was detectable in the presence of pyruvate or glycollic acid.

Preliminary identification of the product of the enzymatic oxidation of D-glycerate as hydroxypyruvate using the procedure of Robins, et al. (1956) was accomplished. This method involved detection of hydroxypyruvate via 3-quinolylhydrazone formation. The experiment included a glycerate dehydrogenase reaction mixture and a reaction mixture control with no substrate or NADP added; both were incubated at room temperature. Sodium phosphate buffer, 0.5 M and pH 11.1, replaced the 0.5 M sodium carbonate buffer, pH 10.5, normally used for assay of glycerate dehydrogenase activity. This prevented interference with the test by the release of carbon dioxide due to acidification of samples. Samples (0.2 ml) removed from both incubation mixtures at zero time, 30 minutes and 60 minutes were used in the 3-quinolylhydrazine determination of α-keto acids. At the same time the course of the enzymatic oxidation was followed spectrophotometrically on the Cary 14 dual beam recording spectro-

Figure 7. Effect of pH on Glycerate Dehydrogenase Activity

Cultures of PA-1 were grown on glycerol in minimal medium A to an optical density at 540 nm of approximately 0.50. The cells were washed twice with saline and the resultant pellet was suspended in 5 ml of 0.02 M Tris, pH 8.5, and sonicated. The sonicate was centrifuged at 12,500 x g with the resultant cell-free extract used in glycerate dehydrogenase assays according to the procedure described in the text.

The different pH values were obtained by using 0.5 M sodium carbonate buffer at various initial pH values. At the higher pH's, the pH of the reaction mixture was adjusted on a Coleman Model 39 pH meter before the addition of NADP. The pH of the reaction mixture was again checked on the pH meter upon completion of the assay.

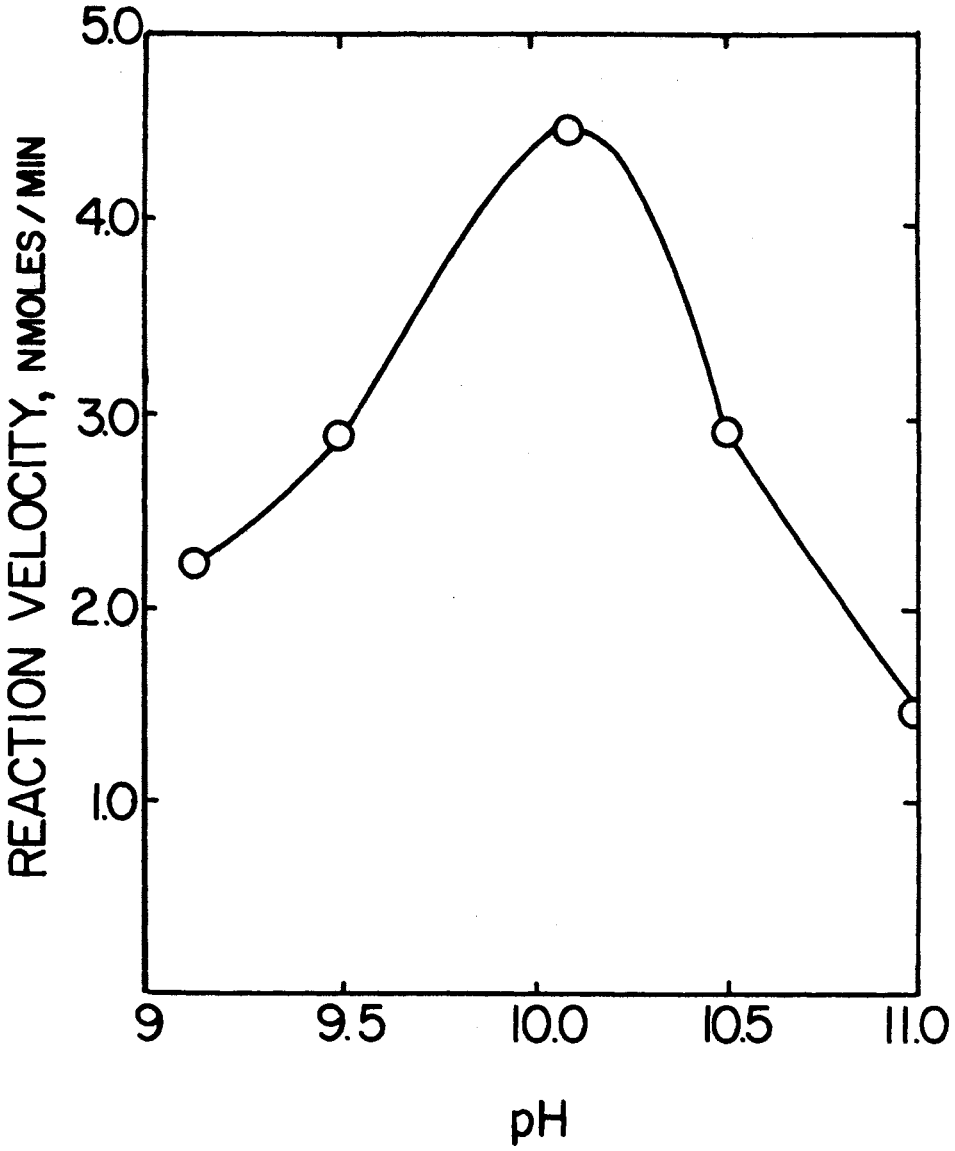

5.0
4.0
3.0
2.0
1.0
REACTION VELOCITY, NMOLES/MIN
9
9.5
10.0
10.5
11.0
pH

TABLE XI

SUBSTRATE SPECIFICITY OF GLYCERATE DEHYDROGENASE ASSAY

Substrate (50 μmoles)	Specific Activity
Glyceric acid	4.84
Sodium glyoxylate	0.29
Sodium lactate	0.15
Sodium butyrate	0.29
β-Hydroxybutyrate	0.59
Sodium pyruvate	0
Glycollic acid	0

Specific activities are nmoles/min/mg protein.

photometer.

The keto acids in the samples were determined by a modification of the procedure of Robins et al. (1956). Two-tenths of a milliliter of 0.5 mM 3-quinolylhydrazine in 0.67 N HCl was added to each 0.2 ml sample immediately after its removal from the reaction mixture and incubated at room temperature for 1 hour. By using 0.5 mM 3-quinolylhydrazine in 0.67 N HCl, a pH of approximately 2.0 was reached when the reagent was added to the 0.2 ml sample. A pH of 1.9-2.2 is optimal for the 3-quinolylhydrazine-α-keto acid reaction. At the end of the one hour incubation period, 2 ml of 0.01 N HCl were added to each sample and the optical density of the solutions was read at 305 nm on the Cary 14 dual beam recording spectrophotometer.

A standard curve for hydroxypyruvate using the 3-quinolylhydrazine test was produced using 0.01 μmole to 2.0 μmoles of hydroxypyruvate. The spectrum of the hydroxypyruvate-3-quinolylhydrazone exhibited a maximum absorbance at 305 nm.

The glycerate dehydrogenase reaction mixture produced 0.05 μmoles of an α-keto acid within 30 minutes with little subsequent increase in α-keto acid at 60 minutes. This level of α-keto acid was approximately one-half the level of hydroxypyruvate produced as calculated from the glycerate dehydrogenase activity followed on the Cary 14 dual beam recording spectrophotometer. This would seem to indicate that the hydroxypyruvate is relatively unstable in the reaction mixture. Sprinson and Chargaff (1946) found that hydroxypyruvic acid underwent a series of rapid and complex tautomeric changes and condensations in 0.01 N sodium hydroxide. With the alkaline pH of the reaction mixture, similar changes could be occurring with the hydroxypyruvate produced by enzyme action.

An alternate method of identification of hydroxypyruvate as the product was attempted, using a procedure derived from Dawkins and Dickens (1965) and Neish (1957). This procedure involved formation of the 2,4-dinitrophenylhydrazone of hydroxypyruvate upon termination of the reaction by deproteinization. Unfortunately, it was not successful due to interference with deproteinization and subsequent dinitrophenylhydrazone extraction by the sodium carbonate or sodium phosphate buffer used in the reaction mixture.

Attempts to demonstrate production of hydroxypyruvate with radioisotopes were unsuccessful. The radioactive sodium glycerate obtained from Calbiochem contained a radioactive contaminant with the same R_f as hydroxypyruvate in the solvent systems used for chromatography. Since glycerate dehydrogenase activity was usually quite low in cell-free extracts, the amounts of hydroxypyruvate generated would be low and easily masked by the radioactive contaminant.

If hydroxypyruvate is the end product of enzymatic oxidation of glyceric acid, reduction of hydroxypyruvate in the presence of cell-free extract containing glycerate dehydrogenase activity should be possible. Using parameters that Dawkins and Dickens (1965) established for the reduction of hydroxypyruvate in the presence of D-glycerate dehydrogenase from rat liver, an assay was developed which allowed detection of enzymatic reduction of hydroxypyruvate using cell-free extract from glycerol-grown PA-1 cells. Incubation of the cell-free extract in an $80^{\circ}C$ water bath for 30 minutes caused a complete loss of hydroxypyruvate reducing activity, verifying enzymatic reduction of hydroxypyruvate.

The reaction mixture for assay of reduction of hydroxypyruvate by glycerate dehydrogenase included: 0.7 ml of 0.2 M sodium phosphate

buffer, pH 6.0; 0.1 ml of 0.001 M $NADH_2$; 0.1 ml of 0.5 per cent lithium hydroxypyruvate; and 0.1 ml of cell-free extract. The controls for this assay are shown in Table XII. There was a high level of endogenous $NADH_2$ oxidase activity, verifying a similar observation with the D-glyceraldehyde enzymatic reduction assay performed under similar conditions. Cell-free extract and $NADH_2$ were included in both the reaction mixture and the blank to compensate for this endogenous activity. Since hydroxypyruvate did not interact with either cell-free extract or $NADH_2$ to cause a decrease in absorbance at 340 nm, hydroxypyruvate was added to the reaction mixture to start the assay. Experiment 2 in Table XII demonstrated that $NADPH_2$ was over 10 times as effective as $NADH_2$ as the coenzyme. Since $NADPH_2$ was not available until late in this investigation, $NADH_2$ was used in most assays.

The substrate specificity of the hydroxypyruvate reduction assay was determined and is shown in Table XIII. Hydroxypyruvate was rapidly reduced by the enzyme while glyoxylate, pyruvate, dihydroxyacetone and pyruvate were only slowly reduced. The much lower level of glyoxylate and pyruvate reduction supports the contention that this enzyme is glycerate dehydrogenase rather than a glyoxylate reductase or lactic dehydrogenase. Kohn and Jakoby (1968) reported that both of the latter enzymes were capable of low level reduction of hydroxypyruvate.

Hydroxypyruvate Dehydrogenase

During the course of this investigation an assay was developed

TABLE XII

COMPONENT REQUIREMENTS FOR THE ASSAY OF THE REDUCTION OF HYDROXYPYRUVATE BY GLYCERATE DEHYDROGENASE

Reaction Cuvette Components	Blank Cuvette Components	Component Omitted From Blank	nmoles of $NADPH_2$ or $NADH_2$ Oxidized/min
Expt. 1			
$NADH_2$ + HP	$NADH_2$	HP	0
CFE + HP	CFE	HP	0
$NADH_2$ + CFE	$NADH_2$	CFE	9.67
$NADH_2$ + HP + CFE	$NADH_2$ + CFE	HP	8.06
Expt. 2			
$NADH_2$ + HP + CFE	$NADH_2$ + CFE	HP	3.22
$NADPH_2$ + HP + CFE	$NADPH_2$ + CFE	HP	33.9

Abbreviations: CFE, cell-free extract; HP, hydroxypyruvate; $NADH_2$, reduced nicotinamide adenine dinucleotide; $NADPH_2$, reduced nicotinamide adenine dinucleotide phosphate.

Seven-tenths of a milliliter of 0.2 M sodium phosphate buffer, pH 6.0, was present in each cuvette.

TABLE XIII

SUBSTRATE SPECIFICITY OF ASSAY FOR REDUCTION OF HYDROXYPYRUVATE

Substrate (50 μmoles)	Specific Activity
Lithium hydroxypyruvate	3.11
Sodium glyoxylate	0.46
Sodium pyruvate	0.23
Dihydroxyacetone	0.12
Sodium potassium tartrate	0.35
Glycollic acid	0

Specific activities are nmoles/min/mg protein.

which would presumably measure hydroxypyruvate dehydrogenase activity in cell-free extract from glycerol-grown PA-1 cells. Such an enzyme could oxidize the hydroxypyruvate generated by oxidation of glyceric acid, producing carbon dioxide and glycolaldehyde (Lamanna and Mallette, 1965). The assay reaction mixture contained: 0.7 ml of 0.5 M sodium carbonate buffer, pH 9.5; 0.1 ml of 0.02 M NAD; 0.1 ml of 0.05 per cent lithium hydroxypyruvate and 0.1 ml of cell-free extract.

The controls for the above assay are presented in Table XIV. There was a significant level of endogenous NAD-reducing activity; therefore, cell-free extract and NAD were included in both the reaction mixture and blank. Since hydroxypyruvate did not cause any increase in absorbance at 340 nm in the presence of either NAD or cell-free extract, it was added to the reaction mixture to start the assay. Hydroxypyruvate was used at a concentration of 0.005 per cent in the reaction mixture since 0.05 per cent in the presence of NAD either caused a very rapid reduction of the NAD or reacted with NAD to produce a product which absorbs light at 340 nm. Burton and Kaplan (1954) had previously noted a slight reaction of hydroxypyruvate with NAD in the presence of hydroxyl ions to produce a product which absorbs light at 340 nm. At a concentration of 0.005 per cent in the reaction mixture, there was very little interaction between NAD and hydroxypyruvate. NAD was a more effective coenzyme than NADP as shown in Table XIV.

Attempts to prove by heat inactivation that the hydroxypyruvate dehydrogenase activity was enzymatic failed. There was no loss in activity either by heating 30 minutes in an 80°C water bath or 10 minutes in a 100°C water bath. However, this activity was only present when both NAD and cell-free extract were mixed with hydroxypyruvate. Optimum

TABLE XIV

COMPONENT REQUIREMENTS FOR THE INTERACTION OF HYDROXYPYRUVATE WITH NAD AND CELL-FREE EXTRACT

Reaction Cuvette Components	Blank Cuvette Components	Component Omitted From Blank	nmoles of NAD or NADP Reduced per Minute
CFE	H_2O	CFE	0
CFE + NAD	CFE	NAD	0.48
CFE + NAD + HP	CFE + HP	NAD	2.58
HP	H_2O	HP	0
HP + CFE	CFE	HP	0
HP + NAD	NAD	HP	0
NAD + CFE + HP	NAD + CFE	HP	2.74
NADP + CFE + HP	NADP + CFE	HP	2.10

Abbreviations: CFE, cell-free extract; HP, hydroxypyruvate; NADP, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide.

Seven-tenths of a milliliter of 0.5 M sodium carbonate buffer, pH 9.5, was present in each cuvette.

activity was present at approximately pH 9.5 with much lower activity present at either pH 9.0 or 10.0.

Enzymes of the Nonphosphorylated Pathway for Glycerol Metabolism in a Glycerol-Negative Mutant

The glycerol-negative mutant of PA-1, GA-73, has been shown by Brown (1972) to be blocked in the phosphorylated pathway for glycerol catabolism. This mutant was found by Brown to have glycerol transport and L-α-glycerophosphate dehydrogenase activities but to lack glycerol kinase activity. Since this mutant was blocked in the phosphorylated pathway for glycerol catabolism, it was examined for enzymes of the nonphosphorylated pathway for glycerol metabolism to determine whether an additional enzyme deficiency there would explain the inability of GA-73 to grow on glycerol as its sole carbon source.

Since GA-73 could not use glycerol as its sole source of carbon, the mutant was grown in minimal medium A containing glucose, which it was capable of growing on, and glycerol to induce the enzymes of the nonphosphorylated pathway for glycerol metabolism. This method of cultivation was known to induce enzymes of the phosphorylated pathway (Brown, 1972). Results of analysis of cell-free extracts of GA-73 for the enzymes of the nonphosphorylated pathway for glycerol metabolism are presented in Table XV. Both the oxidizing and reducing activities of the enzymes of the nonphosphorylated pathway investigated in the present study were present in the mutant. It was necessary, however, to use 0.1 ml of 80 per cent glycerol as the substrate for detection of the low level of glycerol dehydrogenase oxidizing activity. A high level of

TABLE XV

ACTIVITIES OF THE ENZYMES OF THE NONPHOSPHORYLATED PATHWAY FOR GLYCEROL IN GLYCEROL-NEGATIVE MUTANT, GA-73

Enzyme	Specific Activity	
	Oxidizing Activity	Reducing Activity
Glycerol dehydrogenase	1.10	39.8
Aldehyde dehydrogenase	2.54	4.42
Glycerate dehydrogenase	1.88	5.52
Hydroxypyruvate dehydrogenase	2.10	--

Specific activities are nmoles/min/mg protein.

D-glyceraldehyde reducing activity was present but the activity in this direction was only about 10 per cent of that of PA-1. The inability of this glycerol-negative mutant to utilize glycerol as its sole source of carbon could not be explained by a lack of one of these enzymes, since all were present at detectable levels.

Factors Related to the Regulation and Control of the Nonphosphorylated Pathway for Glycerol Metabolism

Oxidizing vs. Reducing Activities of Enzymes

Since the enzymes of the nonphosphorylated pathway in PA-1 had been measured in different extracts at different times, it seemed desirable to measure levels of oxidizing and reducing activities of all enzymes in a single extract. For this experiment, PA-1 was grown in minimal medium A with glycerol as the only carbon source. The results of enzyme assays using a single cell-free extract are presented in Table XVI. The level of reducing activity was much higher than the level of oxidizing activity for each enzyme, which agrees with the results obtained with the mutant GA-73 when it was grown on glucose plus glycerol. It should be pointed out that $NADPH_2$ was used as the coenzyme for detection of glycerate and aldehyde dehydrogenase oxidizing activities in this experiment while the coenzyme $NADH_2$ was used in the GA-73 analysis. Therefore, levels of these two enzymes are probably comparable in the two strains. As has been previously noted in this chapter, glycerate and aldehyde dehydrogenases give approximately 10 times greater levels of reducing activity with $NADPH_2$ than with $NADH_2$. The levels of activity in the mutant could also be affected by catabolite repression (see below).

TABLE XVI

ACTIVITIES OF ENZYMES OF THE NONPHOSPHORYLATED PATHWAY FOR GLYCEROL IN WILD-TYPE CELLS

Enzyme	Specific Activity	
	Oxidizing Activity	Reducing Activity
Glycerol dehydrogenase	4.76	359
Aldehyde dehydrogenase	4.33	60.8
Glycerate dehydrogenase	4.88	27.6

Specific activities are nmoles/min/mg protein.

Due to the higher reducing activities of the enzymes for the nonphosphorylated pathway for metabolism of glycerol and their high levels of activity near pH 7, it may be that this pathway is generally used in synthesis of glycerol rather than in degradation.

Inducibility or Constitutivity of the Enzymes

Cell-free extracts of PA-1 grown on different carbon sources were examined for the presence of enzymes of the nonphosphorylated pathway for glycerol metabolism to determine whether the enzymes were inducible or constitutive. The results of these determinations are presented in Table XVII. Glycerol dehydrogenase and possibly glycerate dehydrogenase are induced when PA-1 is grown on glycerol. Levels of glycerol dehydrogenase were quite low in all extracts except that from glycerol-grown cells. Glycerate dehydrogenase was approximately 3-fold higher in glycerol-grown cells. Aldehyde dehydrogenase is not induced by growth of PA-1 on glycerol nor is the "hydroxypyruvate dehydrogenase" activity. The high level of aldehyde dehydrogenase present in ethanol-grown cells may represent the inducible paraconstitutive aldehyde dehydrogenase of Tigerstrom and Razzell (1968).

Catabolite Repression of Glycerol Dehydrogenase

In previous studies of the phosphorylated pathway for glycerol degradation in P. aeruginosa (Tsay, 1971; Brown, 1972), induction of the enzymes was accomplished by growth of the cells on medium containing both glucose and glycerol or by growth on glucose followed by exposure to glycerol for four hours. These methods were used since they could be applied to both wild-type cells and mutants unable to grow on glycerol.

TABLE XVII

INDUCIBILITY OF ENZYMES

Enzyme	Specific Activity				
	Glucose	Glycerol	Ethanol	Lactate	Succinate
Glycerol dehydrogenase	0.69	3.45	0.10	0.45	0.45
Aldehyde dehydrogenase	3.23	1.93	22.1	6.36	2.44
Glycerate dehydrogenase	1.15	3.41	1.33	1.33	1.33
Hydroxypyruvate dehydrogenase	2.59	1.65	1.23	2.66	2.93

Specific activities are nmoles/min/mg protein.

Column headings indicate the compound used as sole carbon source for growth of cells used to prepare extracts for enzyme assays.

When the same methods were used with PA-1 early in the present study to induce glycerol dehydrogenase, they were unsuccessful. Levels of glycerol dehydrogenase in cells grown on glucose plus glycerol were barely detectible. The enzyme was induced in PA-1 grown on pyruvate plus glycerol but not in cells grown on pyruvate alone. However, no induction occurred in the mutant, GA-73, under the same conditions. This observation cannot be explained on the basis of present knowledge, but it precluded the use of this medium for induction. When PA-1 was grown on glucose, then exposed to glycerol for four hours, no induction occurred. A study of the time required for induction under these conditions was made, with enzyme levels being determined at 30, 60, 120, 180, 240 and 480 minutes, and it was found that glycerol dehydrogenase activity began to increase slightly only at 8 hours. From these studies, it appeared that glycerol dehydrogenase might be subject to catabolite repression in the presence of glucose and that recovery from repression was quite slow, possibly indicating the necessity of diluting out or degrading an inhibitory metabolite before induction could occur. Therefore, an experiment was devised to test this possibility.

Induction of the enzymes of the nonphosphorylated pathway for glycerol metabolism after growth on glucose was studied by first growing PA-1 in the presence of glucose to an optical density at 540 nm of approximately 0.50, harvesting and washing the cells twice with saline at room temperature, and resuspending the cells in minimal medium A containing glycerol to give the initial optical densities shown in Table XVIII. These freshly inoculated cultures were then grown to an optical density of 0.50 and cell-free extracts were prepared and analyzed for the enzymes of the nonphosphorylated pathway for glycerol metabolism. To determine

TABLE XVIII

CATABOLITE REPRESSION OF GLYCEROL DEHYDROGENASE

Enzyme[1]	Specific Activity	Cell Counts: Time of Inoculation	Cell Counts: Time of Harvesting	Number of[2] Cell Divisions
Glycerol dehydrogenase				
(0.05)	3.94	1.4×10^8	2.3×10^9	4.1
(0.10)	3.27	2.6×10^8	2.1×10^9	3.0
(0.15)	1.97	5.7×10^8	2.4×10^9	2.1
Aldehyde dehydrogenase				
(0.05)	2.15			
(0.10)	2.11			
(0.15)	2.52			
Glycerate dehydrogenase				
(0.05)	5.11			
(0.10)	3.62			
(0.15)	3.15			

[1]Numbers in parentheses represent the initial optical density at 540 nm of PA-1 in minimal medium A plus glycerol.

[2]Number of cell divisions (generations) = $\frac{\log N_1 - \log N_o}{\log 2}$.

Specific activities are nmoles/min/mg protein.

the number of cell divisions (or generations) each culture went through, viable counts were made of each glycerol culture at the times of inoculation and of harvesting by aseptically removing a sample, serially diluting with sterile 0.85 per cent saline and plating on nutrient agar. The results of this experiment are presented in Table XVIII.

The glycerol dehydrogenase did appear to exhibit catabolite repression. Aldehyde dehydrogenase was unaffected and glycerate dehydrogenase was partially repressed by growth on glucose. The low level of inoculum in the glycerol culture with initial optical density of 0.05 allowed 4 generations in reaching an optical density of 0.50, while the higher levels of inocula in the other two cultures allowed 3 and 2 generations, respectively. The levels of glycerol dehydrogenase activity in each of the three cultures were proportional to the number of generations after removal from glucose. The higher number of cell divisions in the culture initiated at 0.05 optical density would have allowed more dilution by cell division of catabolic compounds accumulated from growth on glucose, thereby relieving the catabolite repression of glycerol dehydrogenase.

CHAPTER IV

DISCUSSION

At the outset of this investigation, two observations of Brown (1972) were of primary importance. One was the detection of glycerol dehydrogenase activity in P. aeruginosa, strain PA-1, which had to be confirmed to warrant this investigation. The other was the release of $^{14}CO_2$ from ^{14}C-glycerol, which Brown observed when a glycerol-negative mutant, GA-73, was grown in glycerol plus glucose and exposed to ^{14}C-glycerol. Brown had suggested generation of the $^{14}CO_2$ by pathway 4 in Figure 1, followed by reactions 1 through 3 below.

$$\text{Hydroxypyruvate} \longrightarrow \text{Serine} \qquad (1)$$

$$\text{CDP-Diglyceride} + \text{Serine} \longrightarrow \text{Phosphatidylserine} \qquad (2)$$

$$\text{Phosphatidylserine} \xrightarrow{CO_2} \text{Phosphatidylethanolamine} \qquad (3)$$

The following reaction (Lamanna and Mallette, 1965; Callely and Dagley, 1959) could also be responsible for the generation of $^{14}CO_2$ observed by Brown and would also follow pathway 4 in Figure 1 (also see Figure 2).

$$\text{Hydroxypyruvate} \xrightarrow{CO_2} \text{Glycolaldehyde} \qquad (4)$$

Cowen (1968) determined that PA-1 was able to grow on glycerol as

the sole carbon source by utilization of the lower portion of the EMP pathway, entering the pathway at the level of triose phosphate. Brown (1972) demonstrated the absence of glycerol kinase in the glycerol-negative mutant, GA-73, thus explaining its inability to grow in glycerol as its sole carbon source. This then posed the question of the reactions through which $^{14}CO_2$ was generated by this mutant from ^{14}C-glycerol, since it could not be generated by metabolic pathways known to occur in P. aeruginosa.

Confirmation of the presence of glycerol dehydrogenase in PA-1 and GA-73 was accomplished using a modification of the assay procedure used by Brown (1972) and Ding (1972). This assay was modified by increasing the concentration of the substrate, glycerol, used to initiate the assay and optimizing the pH at which the assay was run. Brown used 0.01 M glycerol in the reaction mixture, which gave very low levels of activity. In this investigation it was found that glycerol at 0.1 M in the reaction mixture gave much higher levels of activity, and that 1.0 M concentrations of glycerol in the reaction mixture gave still higher levels of activity. Sheys and Doughty (1971) presented a theory to explain why high concentrations of ethyl alcohol were required to detect alcohol dehydrogenase activity in Rhodotorula; the same explanation might apply to glycerol dehydrogenase. These authors postulated that for alcohol dehydrogenase the rate-limiting step was dissociation of the enzyme-NADH binary complex. At high alcohol concentrations, an abortive enzyme-NADH-alcohol ternary complex could be formed from which NADH dissociated more rapidly. An alternative explanation for the glycerol dehydrogenase would be that under cellular physiological conditions the enzyme may function much more effectively in the reverse direction than in the

forward direction, thereby requiring very high concentrations of substrate to move it in the forward direction. This theory seems to be supported by the much higher level of enzyme activity in the reverse direction and an almost total converison of ^{14}C-glyceraldehyde to ^{14}C-glycerol.

The pH optimum for glycerol dehydrogenase activity is quite high, i.e., 10.7; however, it agrees closely with the pH optimum of 10.0 determined for glycerol dehydrogenase from E. coli by Asnis and Brodie (1953). They explained the high pH optimum by the fact that all reactions involving oxidation-reduction of nicotinamide adenine dinucleotides are of the following type:

$$\text{Substrate-H}_2 + \text{NAD} \longrightarrow \text{Substrate} + \text{NADH} + \text{H}^+$$

Since hydrogen ions are involved in the reaction, an increasingly alkaline pH would favor shifting of the equilibrium to the right and the activity of the enzyme would increase with increasing pH up to the point at which the high alkaline pH begins to denature the enzyme. The fact that the reduction of D-glyceraldehyde by the glycerol dehydrogenase proceeds much more rapidly at pH 6.0 than at high pH values supports the above theory, since a more acid pH would favor a shift of the equilibrium to the left. The enzyme denaturation at the high alkaline pH would also explain the rapid decrease in glycerol dehydrogenase activity once its pH optimum had been surpassed. The activity of glycerol dehydrogenase across a broad pH range seems to be typical for similar enzymes from a variety of sources (Asnis and Brodie, 1953; Goldman, 1963; Bosron and Prairie, 1972).

All enzyme assays in this investigation were accomplished using

unfractionated cell-free extract and measuring reduction or oxidation of pyridine nucleotide. Therefore, it cannot be stated with certainty whether results from examination of substrate specificity of an enzyme assay represent specificity of a single enzyme or activities of more than one enzyme with possibly overlapping or different specificities, which are active under the same assay conditions. However, it seemed worthwhile to determine the range of substrates which could be oxidized or reduced under each set of assay conditions employed. This would allow comparison with substrate specificities of similar enzymes from other organisms. If the glycerol dehydrogenase activity as measured in the present study does represent the activity of a single enzyme, its substrate specificity was shown to be quite general in both oxidizing and reducing capacity. The substrate specificity of the glycerol dehydrogenase is in general agreement with that of similar enzymes from pig kidney cortex (Bosron and Prairie, 1972), rabbit skeletal muscle (Kormann et al., 1972), and pig brain (Turner and Tipton, 1972).

The reduction of D-glyceraldehyde by the glycerol dehydrogenase was shown to be approximately 75 times the oxidizing activity of the enzyme from the same cell-free extract (Table XVI). This is typical of enzymes performing similar functions in other organisms (Bosron and Prairie, 1972; Kormann et al., 1972; and Moore, 1959).

The product of the oxidation of glycerol by glycerol dehydrogenase was identified as D-glyceraldehyde via the Sawicki test for aliphatic aldehydes and finally by using semicarbazide as a trapping agent for D-glyceraldehyde. The D-glyceraldehyde was difficult to detect not only due to the slow rate of oxidation of glycerol by glycerol dehydrogenase, but also to the ease with which glyceraldehyde can react with itself

and other compounds at an alkaline pH. Gutsche et al. (1967) were able to show that the equilibrium constant for a mixture of D-glyceraldehyde and dihydroxyacetone was approximately 17 (in favor of dihydroxyacetone) at an alkaline pH and also that the mixture of these two compounds produced condensation products which included fructose, sorbose and dendroketose. Since the pH of the glycerol dehydrogenase reaction mixtures was at least 8.5 in the case of semicarbazone formation and usually 10.7 for all other assays, the difficulty encountered in attempts to identify the radioactive product formed from ^{14}C-glycerol by chromatography is readily understandable.

With the identification of D-glyceraldehyde as the product formed from glycerol by glycerol dehydrogenase, it is possible to state that a new enzyme, a glycerol dehydrogenase which produces D-glyceraldehyde, has been found in P. aeruginosa. A glycerol dehydrogenase of this type has not previously been reported in bacteria. All bacterial glycerol dehydrogenases up to the time of this investigation oxidized glycerol to produce dihydroxyacetone instead of D-glyceraldehyde (Hauge et al., 1955; Asnis and Brodie, 1953; Burton and Kaplan, 1953; and Goldman, 1963) and were capable of reducing dihydroxyacetone which this enzyme is not. The fact that the isomerization of D-glyceraldehyde to dihydroxyacetone proceeds readily while the reverse reaction does not, precludes the possibility that the true product of the glycerol dehydrogenase reaction is dihydroxyacetone.

The confirmation of the presence of glycerol dehydrogenase activity in P. aeruginosa strengthened the possibility that glycerol could be metabolized via the route suggested by Brown (1972). The next enzyme in this pathway would be a glyceraldehyde dehydrogenase or a generalized

aldehyde dehydrogenase. Another possibility for further metabolism of glycerol would be phosphorylation of D-glyceraldehyde by a kinase. No D-glyceraldehyde kinase activity was detectable in PA-1, but a dehydrogenase for D-glyceraldehyde was found. The enzyme appeared to be a generalized aldehyde dehydrogenase since cell-free extract oxidized a variety of aldehydes quite readily under the conditions of the assay. General aldehyde dehydrogenases have been reported in P. aeruginosa (Tigerstrom and Razzell, 1968) as well as in other organisms (Feldman and Weiner, 1972; Steinman and Jakoby, 1968; and Jakoby, 1958). The aldehyde dehydrogenase of glycerol-grown PA-1 appeared to be quite different from the aldehyde dehydrogenase reported in ethanol-grown P. aeruginosa by Tigerstrom and Razzell (1968) in several ways. P. aeruginosa is known to have a variety of aldehyde dehydrogenases (Tigerstrom and Razzell, 1968; Jakoby, 1963; Heydeman and Azoulay, 1963). Although the pH optima of the aldehyde dehydrogenases from the two sources were similar, the aldehyde dehydrogenase from ethanol-grown cells exhibited activity over a much broader pH range than the aldehyde dehydrogenase from glycerol-grown cells. In addition the aldehyde dehydrogenase of glycerol-grown cells did not exhibit the substrate inhibition that was reported for the aldehyde dehydrogenase from ethanol-grown cells by Tigerstrom and Razzell (1968). The substrate specificities of the two enzymes were quite different. There was also a significant difference in the heat stability of the enzymes from the two different sources. Finally, the aldehyde dehydrogenase of Tigerstrom and Razzell had very little activity (8 per cent) in the absence of potassium ions, while the aldehyde dehydrogenase from glycerol-grown cells had no requirement for potassium ion.

The observed reduction of D-glycerate by extract from glycerol-grown cells and the lack of such activity in extract from ethanol-grown cells also suggests that the aldehyde dehydrogenases are two different enzymes if the reduction is due to aldehyde dehydrogenase. It is possible that the oxidation of aldehydes is mediated by one enzyme and the reduction of D-glycerate by another, and that the ethanol-grown cells simply lack the latter enzyme. In general, aldehyde dehydrogenases are not reversible (Seegmiller, 1955; Black, 1955; Racker, 1955); however, Stadtman and Burton (1955) have reported a reversible aldehyde dehydrogenase from Clostridium kluyveri which produces acetyl coenzyme A from acetaldehyde. The question of whether the oxidizing and reducing activity of the proposed aldehyde dehydrogenase represent a single enzyme or two different enzymes can only be resolved by purification of the aldehyde dehydrogenase or possibly of more than one aldehyde dehydrogenase.

One interesting note regarding the reduction of D-glycerate is the fact that high levels of activity occur at pH 9.0, which is contrary to the theory presented above that reduction in the presence of nicotinamide adenine dinucleotides should be favored under acidic conditions. The reducing activity of the enzyme was approximately 15 times the oxidative activity as shown in Table XVI.

Great difficulty was encountered in determining the products of aldehyde dehydrogenase action in either direction because of the interaction of D-glyceraldehyde and D-glycerate with components of the reaction mixture, which resulted in poor separation of isotopically labelled compounds during chromatography, particularly when the reduction of D-glycerate was involved. Evidence was obtained by chromatography of labelled product that D-glycerate was the product of oxidation of

D-glyceraldehyde by the enzyme although further proof is essential.

A D-glycerate dehydrogenase was found in glycerol-grown cells while no D-glycerate kinase was detectable. The oxidizing activity of this enzyme had an alkaline pH optimum comparable to that of D-glycerate dehydrogenases from other sources (Sugimoto et al., 1972; Kohn and Jakoby, 1968). The enzyme was shown to be relatively specific for D-glycerate which is typical for glycerate dehydrogenases (Kohn and Jakoby, 1968; and Heinz et al., 1962). Hydroxypyruvate was tentatively identified as the product of oxidation of D-glycerate by the procedure of Robins et al. (1956).

The reducing activity of this enzyme was found to be higher than its oxidizing activity as shown in Tables XV and XVI. Dawkins and Dickens (1965) have shown that the D-glycerate dehydrogenase of rat liver also has a higher level of reducing than oxidizing activity. The enzymatic reduction of D-glycerate, presumably by the D-glycerate dehydrogenase, is specific for hydroxypyruvate as was the reducing activity of a D-glycerate dehydrogenase from P. acidovorans (Kohn and Jakoby, 1968).

According to Brown's suggested pathway the hydroxypyruvate produced from glycerate dehydrogenase should next be converted to serine. No evidence was sought for this in the present study; however, the possibility of a hydroxypyruvate dehydrogenase action resulting in decarboxylation and production of glycolaldehyde as suggested by Lamanna and Mallette (1965) was explored. There was apparent hydroxypyruvate dehydrogenase activity present; however, this enzyme activity was quite heat resistant. It is possible that such an enzyme could be heat resistant, as Dixon and Webb (1964) have documented for other enzymes.

The activity was only measurable when both hydroxypyruvate and cell-free extract were present, which indicates that it is enzymatic. If this reaction does take place, reaction four given at the beginning of this chapter could generate the carbon dioxide observed by Brown.

As shown in Tables XV and XVI, all of the enzymes of the nonphosphorylated pathway for glycerol are present in both the glycerol-negative mutant, GA-73, and PA-1. This would allow the generation of CO_2 by the glycerol-negative mutant by Brown's suggested pathway. It should be pointed out that this mutant is unable to grow on glycerol as its sole carbon source; therefore, this pathway for glycerol metabolism can not be a major pathway for production of biologically useful energy or of precursors for all cellular syntheses. It is possible that this is a pathway for production of certain cellular constituents such as L-serine and phosphatidylethanolamine for cellular protein and phospholipid, respectively. Phosphatidylethanolamine has been shown to make up 69 per cent of the lipid in P. aeruginosa (Randle et al., 1969).

This pathway may also function in the synthesis of glycerol from other short-chain carbon compounds such as glycine, glycollic acid, oxalic acid, glyoxylic acid, L-tartrate, ethanol and methanol (Kornberg and Gotto, 1959; Kohn and Jakoby, 1968; Dunstan et al., 1972; Harder and Quayle, 1971), when the EMP pathway is not functioning normally. The higher rate of reduction by the enzymes of the nonphosphorylated pathway would suggest that this may be the case. Sugimoto et al. (1972) had evidence which indicated that L-serine could be catabolized to a glycolytic intermediate via glycerate dehydrogenase. If synthesis of glycerol from triose phosphate is blocked, as in other glycerol mutants studied by Brown (AH-8), it might be possible to use pathway 4 in Figure 1 to

produce glycerol from L-serine. It is possible that the higher level of reducing activity of the enzymes would be unimportant, however, if one of the enzymes, for example, hydroxypyruvate dehydrogenase, were irreversible, causing the pathway to function in the oxidation of glycerol.

There is also the possibility that these enzymes are not all a normal part of a single pathway. It is possible that one or more of the enzymes is normally used to act on a closely related substrate in a completely different pathway. The glycerol dehydrogenase may actually function within the cell as a sorbitol, ribitol or 1,2-propanediol dehydrogenase or as a glyceraldehyde reductase, while the aldehyde dehydrogenase may be functioning to oxidize aldehydes of fatty acids or aromatic compounds. Recently Wu (1973, and personal communication) was able to show enzymes functioning in just such a fashion. Using a strain of E. coli able to utilize 1,2-propanediol but not arabitol as its sole source of carbon, he was able to isolate a mutant capable of utilizing arabitol as sole source of carbon by two consecutive mutations. The first-stage mutant could no longer grow on 1,2-propanediol since its permease had been altered to function as an arabitol permease. The second-stage mutant was constitutive for galactose dehydrogenase for which it had previously been inducible. The galactose dehydrogenase was capable of acting on arabitol, thus explaining the newly gained ability of the mutant to grow on arabitol as its sole source of carbon. Again this points out the ability of cells to utilize enzymes normally used in one pathway for other purposes under different conditions, thus showing how modification or a combination of mutation and modification may enhance survival of an organism in a variety of environments.

There are other ways that CO_2 may be generated from this nonphos-

phorylated pathway for glycerol catabolism. One such means involves the formation of D-erythrulose from hydroxypyruvate in the presence of a yeast carboxylase as Dickens and Williamson (1956) have described. This enzyme-catalyzed reaction is given below.

$$2 \text{ Hydroxypyruvate} \xrightarrow{2CO_2} \text{D-erythrulose} \qquad (5)$$

Horecker et al. (1953) have observed condensation of D-glyceraldehyde with active glycolaldehyde derived from hydroxypyruvate in the presence of spinach transketolase with the release of CO_2 and D-ribulose.

All of the enzymes of the proposed nonphosphorylated pathway were produced in at least low levels during growth of PA-1 on any of the carbon sources listed in Table XVII. Glycerol dehydrogenase and glycerate dehydrogenase were induced to higher levels of activity by growth on glycerol. The high level of aldehyde dehydrogenase produced by growth on ethanol probably represents the inducible paraconstitutive aldehyde dehydrogenase of Tigerstrom and Razzell (1968).

As demonstrated in Table XVIII, apparent catabolite repression of glycerol dehydrogenase and glycerate dehydrogenase occurs in cells grown on glucose and is relieved by subsequent prolonged growth on glycerol. Magasanik (1961) stated that all glucose-sensitive enzymes were capable of converting their substrates to metabolites which the cell could obtain independently and more readily from glucose metabolism. In short the cell would have nothing to gain by the synthesis of enzymes required for glycerol degradation. If this type of reasoning is applied to the results of this investigation, the nonphosphorylated pathway should be involved in production of catabolic products normally produced by the

EMP pathway, closely related pathways, and the citric acid cycle; therefore, it should be able to utilize the pathway for growth even though the phosphorylated pathway is blocked as in GA-73. Since GA-73 can not grow on glycerol as its sole carbon source, this can not be the case. There is still the alternate possibility, however, that the enzymes of this proposed pathway for glycerol metabolism are enzymes which normally function in some other pathway. The pathway they are involved in may function in the production of metabolites which the EMP and citric acid cycle also produce, thereby explaining the repressibility of glycerol dehydrogenase and glycerate dehydrogenase.

Lessie and Neidhardt (1967) observed that the highest levels of Entner-Doudoroff pathway enzymes were induced by growth of P. aeruginosa on glycerol with highest levels of glucose-6-phosphate dehydrogenase being observed in cultures where growth on glycerol was poor. Also Hyeleman and Phibbs (1972) observed induction to maximum levels of many hexose-catabolizing enzymes when P. aeruginosa was grown on glycerol. The above observations could explain why growth of PA-1 on glycerol induces glycerol dehydrogenase and glycerate dehydrogenase if they are normally functional in a pathway related to glucose degradation.

Magasanik (1961) also pointed out that rapid degradation of glucose results in a high intracellular content of adenosine triphosphate and other "energy-rich" compounds. Rosenblum et al. (1971) found that adenosine triphosphate and guanosine triphosphate inhibited beef liver D-glycerate dehydrogenase. This points out the possibility that the high levels of adenosine triphosphate generated by growth on glucose could explain the apparent catabolite repression of glycerol dehydrogenase and D-glycerate dehydrogenase.

Recent studies (Varmus et al., 1970) of the mechanism of catabolite repression in E. coli have shown that 3',5'-adenosine monophosphate (cyclic AMP) is the critical metabolite in that organism. Low levels of cAMP are present during growth on glucose and this prevents synthesis of inducible enzymes since induction requires cAMP. Addition of cAMP to repressed cells relieves catabolite repression. There is no evidence for a similar mechanism in P. aeruginosa, and further investigation of catabolite repression in this organism is needed. Therefore, speculation based upon previously proposed mechanisms may or may not be meaningful.

Further investigation of the proposed nonphosphorylated pathway for glycerol metabolism is warranted. The next most obvious step would be purification of the enzymes thus far implicated. This would allow adequate characterization of the enzymes as well as absolute confirmation of their presence in the cell. In the case of aldehyde dehydrogenase and D-glycerate dehydrogenase, identification of products could be confirmed with the pure enzymes. In addition, study of the effect of adenosine triphosphate and various glycolytic intermediates on the different enzyme activities might provide some information regarding their function.

CHAPTER V

SUMMARY AND CONCLUSIONS

Pseudomonas aeruginosa strain PA-1 has been found to produce enzymes capable of degrading or synthesizing glycerol by a nonphosphorylated pathway. The enzyme activities involved include: glycerol dehydrogenase, aldehyde dehydrogenase, D-glycerate dehydrogenase and probably hydroxypyruvate dehydrogenase.

The glycerol dehydrogenase activity was shown to be generalized with regard to polyalcohols oxidized and aldehydes reduced. Its pH optimum for oxidizing activity was 10.7 and for reducing activity was below 7.0. The enzyme activity was shown to produce D-glyceraldehyde from oxidation of glycerol, and glycerol from reduction of D-glyceraldehyde. The production of D-glyceraldehyde by the oxidizing activity was verified by the benzothiazolone hydrazone test for aliphatic aldehydes, and by trapping with semicarbazide followed by chromatographic identification of the derivative. A modification of the benzothiazolone hydrazone test was also used to detect D-glyceraldehyde on chromatograms. The production of glycerol by enzymatic reduction of D-glyceraldehyde was verified by use of radioisotopes and chromatography.

The aldehyde dehydrogenase activity was shown to be general toward oxidation of aldehydes and relatively specific for reduction of D-glycerate. The pH optimum of the aldehyde dehydrogenase oxidizing activity was 8.5 and of its reducing activity approximately 9.0. The oxidizing

activity of aldehyde dehydrogenase from glycerol-grown cells was shown to be quite different from that from ethanol-grown cells. Differences which were shown included: (1) different substrate specificities, (2) lack of substrate inhibition of the aldehyde dehydrogenase from glycerol-grown cells, (3) differences in effect of pH, (4) differences in heat stability, and (5) a difference in requirement for potassium ion. The product of oxidation of D-glyceraldehyde by aldehyde dehydrogenase has been tentatively identified via radioisotopes and chromatography as D-glycerate.

Aldehyde dehydrogenase from ethanol-grown cells lacked reducing activity. This suggested that the "aldehyde dehydrogenase" oxidizing and reducing activities observed in glycerol-grown cells may actually represent two distinct enzymes.

The glycerate dehydrogenase activity was shown to be quite specific for oxidation of D-glycerate and reduction of hydroxypyruvate. The pH optimum for the oxidizing activity of glycerate dehydrogenase was 10.2, and for the reducing activity was below pH 7.0. The product of oxidation of D-glycerate by glycerate dehydrogenase has been tentatively identified as hydroxypyruvate by use of the 3-quinolylhydrazine test for α-keto acids. The enzymatic formation of a product which reacted with 3-quinolylhydrazine to form a 3-quinolylhydrazine derivative with maximum absorbance at 305 nm was observed.

Hydroxypyruvate dehydrogenase activity has been observed in cell-free extracts from PA-1. The activity is heat-stable but other requirements indicate that it is enzymatic in nature.

All four enzyme activities were found in a glycerol-negative mutant; therefore, a block in this pathway cannot be used to explain the inabil-

ity of the mutant to grow on glycerol as its sole carbon source. The reducing activities of the first three enzymes are much higher than their oxidizing activities, suggesting that the pathway may actually function in glycerol synthesis rather than in degradation. It has been proposed, however, that the reaction catalyzed by hydroxypyruvate dehydrogenase may be irreversible and therefore the pathway could be used in degradation of glycerol.

The enzymes, glycerol dehydrogenase and glycerate dehydrogenase, have been shown to be induced by growth of PA-1 on glycerol while the aldehyde dehydrogenase and hydroxypyruvate dehydrogenase are not. The induction of these enzymes was repressed by prior growth of PA-1 on glucose. It is possible that these enzymes may normally function in other pathways involving the same substrates or closely related substrates. With these enzymes available in the cell, they may be utilized in glycerol metabolism.

With the finding of these enzyme activities, it is proposed that the CO_2 generated from glycerol by the glycerol-negative mutant, GA-73, may be produced by decarboxylation of hydroxypyruvate by a hydroxypyruvate dehydrogenase. Alternate possibilities for generation of CO_2 from this pathway were presented.

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