CATABOLIC PATHWAYS AND METABOLIC CONTROLS IN *PSEUDOMONAS AERUGINOSA*

By

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CATABOLIC PATHWAYS AND METABOLIC CONTROLS

IN PSEUDOMONAS AERUGINOSA

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CHAPTER I

INTRODUCTION

Synthesis of the enzymes of certain catabolic pathways of metabolism is generally recognized to be initiated by the presence of specific inducer molecules which interact with the product of a regulatory gene (the repressor) and determine its functional state (1). In the absence of inducer, the repressor acts on the operator to prevent the functioning of the genes under its control. The inducing molecule renders the repressor inactive in its inhibitory function.

Synthesis of catabolic enzymes may also be subject to the regulatory mechanism which has been labeled "catabolite repression" (2). The end products of biosynthetic pathways are unique and specific, whereas the catabolic pathways are convergent, leading to a relatively restricted group of common catabolites. The metabolism of glucose, and certain other substrates which allow a rapid rate of growth, may be expected to result in an accumulation of catabolites within the cell. Catabolite repression refers to the repression of the synthesis of those enzymes which would tend to increase the concentration of this intracellular pool of compounds.

Recently, data have been presented which show that mechanisms exist for the regulation of enzyme activity in catabolic pathways which are similar to the feedback inhibition of anabolic pathways. Komolrit and Gaudy (3) have shown that cells which were growing

exponentially on various different polyalcohol substrates ceased to utilize them immediately on the addition of glucose to the medium. Resumption of utilization of the original substrate abruptly followed the depletion of glucose. Zwaig and Lin (4) have described a specific case of feedback inhibition in the pathway of glycerol dissimilation in Escherichia coli. Glycerol kinase was found to be subject to inhibition by the glycolytic intermediate, fructose-1,6-diphosphate.

The elucidation of the mechanisms of metabolic control has largely been conducted using E. coli. Genetic data are also available for E. coli and Salmonella typhimurium indicating clustering of related genes (5). No genetic data have been obtained for catabolic pathways in Pseudomonas aeruginosa, however, and comparatively little has been done in the area of control mechanisms for degradative pathways. Mandelstam and Jacoby (6) have examined the enzymes of mandelate degradation in P. fluorescens and found three distinct operons for the eight enzymes of the pathway. The enzymes of each operon are induced as a group by the product of the preceeding operon, and the synthesis of each group of enzymes is "multi-sensitive" to repression by the products of succeeding operons.

A more recent study of the mandelate pathway in Pseudomonas putida (fluorescens) showed similar coordinate control of the three groups of enzymes (7, 8, 9). The first group of five enzymes was found to be coordinately induced not only by the first intermediate of the pathway, but also by benzoylformate, the third intermediate of the sequence. The phenomenon was demonstrated by employing mutants which were specifically blocked so as to be unable either to further degrade benzoylformate or to produce back-induction by its endogenous

conversion to mandelate. A non-metabolizable inducer, phenoxyacetate, was tested with similar results; all five enzymes of the mandelate group were formed simultaneously without induction of the enzymes of the subsequent groups.

The present studies were undertaken as a preliminary to future investigations into the genetic and metabolic control mechanisms of degradative pathways in P. aeruginosa. A general survey of the growth characteristics of the organism on various carbon sources led to the choice of the glycerol pathway for more extensive investigation. The enzymes of the glycerol pathway appeared to be inducible, repressible by glucose, and also subject to inhibition during the metabolism of glucose. Therefore, the pathway appeared to be a fruitful one for the purpose of investigating mechanisms of metabolic control. A necessary first step of such an investigation is the determination of the reactions involved in the pathway.

Two principle pathways for the dissimilation of glycerol have been found among bacteria. These are shown in Figure 1. In one pathway, glycerol is phosphorylated to form glycerophosphate and subsequently oxidized to dihydroxyacetone phosphate (reactions 1 and 2). In the other pathway, glycerol is first oxidized to dihydroxyacetone prior to phosphorylation to triose phosphate (reactions 3 and 4). The former pathway has been demonstrated for Mycobacterium (10), Streptococcus faecalis (10), Escherichia freundii (10), Aerobacter aerogenes (10), and E. coli (11). The latter pathway has been found in Acetobacter suboxydans (10), Aerobacter aerogenes (10), and E. coli (12). The presence of both pathways in A. aerogenes strain 1033 was described by Lin, et al (13). This organism contains an inducible

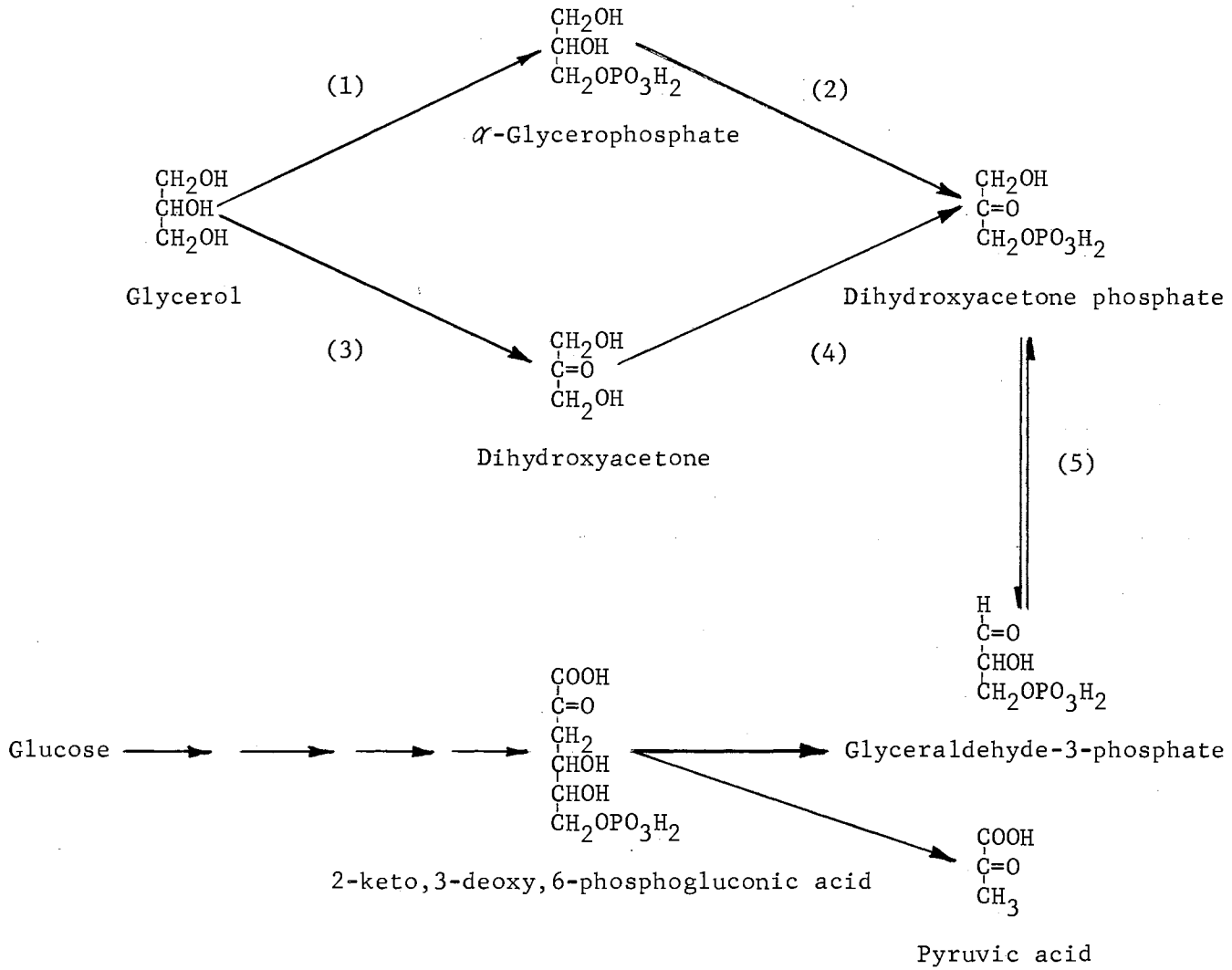


Figure 1. Major Pathways for Glycerol Metabolism in Bacteria

NAD-linked glycerol dehydrogenase when grown on glycerol under anaerobic conditions. When exposed to aerobic conditions, the glycerol dehydrogenase is rapidly destroyed and glycerol is converted to glycerophosphate, followed by oxidation to triose phosphate by a glycerophosphate dehydrogenase which does not require NAD. The electron carrier has not been identified.

Different pathways have also been described for E. coli in independent investigations. Asnis and Brodie (12) reported the conversion of glycerol to dihydroxyacetone by extracts of E. coli, but no data were presented as to the conditions of growth of the cells. More recently, Koch, et al (11), studied the aerobic dissimilation of glycerol in E. coli strain K10. The aerobic pathway was shown to proceed via the glycerophosphate intermediate. These investigators reported the absence of the anaerobic pathway.

A third pathway of metabolism, characteristic of the lactobacilli, involves the transformation of glycerol into β-propionaldehyde, β-hydroxypropionic acid and trimethylglycol (14).

Glycerol has been shown to penetrate bacterial cells readily. Hayashi and Lin (15) have determined that free diffusion of glycerol into cells of E. coli K12 is not rate-limiting for growth even at very low concentrations. No active transport system was found for glycerol, but several mutant organisms were isolated which had lost the wild-type ability to accumulate the labeled substrate. These mutants were further shown to lack glycerol kinase and were therefore unable to "trap" the substrate by its conversion to L-α-glycerophosphate, the latter compound being unable to diffuse freely across the cell membrane.

Experiments by Lin, et al (16), showed that E. coli K10 was capable of using exogenous L-α-glycerophosphate without hydrolysis prior to uptake. This was reported by Hayashi, et al (17), to be due to the presence of a specific transport mechanism for L-α-glycerophosphate. The enzymes of the transport system, glycerol kinase, and L-α-glycerophosphate dehydrogenase were all subject to induction by both glycerol and L-α-glycerophosphate, but an analysis of mutants which were unable to synthesize the particular enzymes revealed that L-α-glycerophosphate was the actual inducer of all three enzymes (11).

In the present studies, cell-free extracts were employed to determine which of the pathways for glycerol metabolism is utilized by P. aeruginosa.

CHAPTER II

MATERIALS AND METHODS

A. Organism

Pseudomonas aeruginosa, strain 1 (PA-1), and the mutants derived from it were used for all studies. The organism was originally obtained from B. W. Holloway of the University of Melbourne.

B. Cultivation Media and Conditions of Growth

The cells were grown at 37° in a minimal medium of M-9 salts (18) containing (g per liter): NH_4Cl, 1.0; $Na_2HPO_4 \cdot 7H_2O$, 11.3; KH_2PO_4, 3.0; NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; distilled water. Carbon sources were autoclaved separately and added to the desired concentration. Two per cent agar was included for the preparation of plates. All liquid cultures were aerated by shaking.

C. Measurement of Growth

Cell suspensions were read against appropriate blanks at 540 mμ on a Coleman Junior Spectrophotometer, Model 6-D.

D. Chemicals

D-glyceraldehyde-3-phosphate (diethylacetal barium salt), disodium DL-α-glycerophosphate, penicillin-G (potassium salt, B grade), nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH),

and adenosine triphosphate (ATP) were obtained from Calbiochem. Muscle α-glycerophosphate dehydrogenase, urocanic acid, and thiazolyl blue (MTT) were products of Nutritional Biochemicals Corporation. N-methyl-N'-nitro-N-nitrosoguanidine was obtained from Aldrich Chemical Corporation. All chemicals used in the preparation of growth media were reagent grade.

E. Chemical Analyses

1. Glucose

Glucose was determined according to the Glucostat method of Worthington Biochemical Corporation. One ml samples containing 0.05 to 0.3 mg glucose were added to 9.0 ml of the prepared Glucostat reagent and allowed to stand at room temperature for exactly 10 minutes. The reaction was stopped by adding one drop of 4M HCl and the tubes were allowed to stand at room temperature at least five minutes prior to reading on a Coleman Junior Spectrophotometer at 400 mμ.

2. Histidine

Histidine was determined by the method described by Jorpes (19). A diazonium solution was prepared by combining 1.5 ml of 5% sodium nitrite solution with 1.5 ml of a solution containing 0.9% sulfanilic acid and 9% HCl. The mixture was cooled on ice for 5 minutes. Then 6.0 ml of the nitrite solution were added with shaking, the solution was cooled again for 6 minutes, and water was added to a volume of 50 ml. Two ml of the diazonium solution were combined with 1.0 ml of the histidine sample (neutral to faintly acid, with histidine at 0.05

to 0.005 mg/ml). After 1 to 3 hours, 5.0 ml of 1.1% sodium carbonate were added. The tubes were read 4 to 8 minutes after the addition of the carbonate at 500 mμ.

3. Glycerol

Komolrit's modification (20) of the method of Neish (21) was used to determine glycerol. Periodate oxidation of glycerol allowed colorimetric determination of the formaldehyde formed. An aliquot containing 0.2 to 0.025 mg of glycerol was made up to 2.0 ml with distilled water and 0.5 ml of freshly prepared 0.1 M periodic acid was added to the sample. Exactly 10 minutes later, 0.5 ml of 1.0 M freshly prepared sodium arsenite was added with thorough mixing. About 10 minutes after addition of the arsenite, 6.9 ml of absolute ethanol were added with thorough mixing. A 1.0 ml sample of that solution was combined with 10.0 ml of chromotropic acid reagent (60% H_2SO_4 containing chromotropic acid at 0.1%) and heated in a boiling water bath for 30 minutes under diffused light. The tubes were cooled to room temperature and read at 570 mμ.

4. Protein

The protein content of cell extracts was determined according to the method of Sutherland, et al (22). The extracts were diluted 1:20 and aliquots made up to 1.0 ml with water. Five ml of a reagent containing sodium carbonate (4%), sodium-potassium tartrate (0.04%), and $CuSO_4$ (0.02%) were added to the protein sample. The tubes were allowed to stand for 40 minutes at room temperature. Phenol reagent, 2 N, (Fisher Scientific Company) was diluted 1:2 with water, 0.5 ml

was added to the tubes and they were mixed immediately. The tubes were read at 660 mμ with appropriate standards.

F. Isolation of Mutants

1. Selection for Mutants Using Nitrosoguanidine and Penicillin

PA-1 was inoculated into 10 ml of nutrient broth and grown to 8 x 10^8 cells/ml. The cells were centrifuged, washed with 10 ml of 0.05 M citrate buffer at pH 6.0, resuspended in 10 ml of citrate buffer which contained nitrosoguanidine at 20 μg/ml (100 μg/ml was used in some experiments), and incubated for 2.5 hours at 37° C with shaking. The cells were harvested by centrifugation, washed with 0.85% NaCl, divided into 10 separate tubes, each containing 6 ml of nutrient broth, and allowed to grow to stationary phase. In some cases, the cells were further subcultured by growing up 0.1 ml of the stationary-phase broth cells to stationary phase in glucose. They were then collected, suspended in saline and incubated for 7 hours with shaking. Tubes were prepared which contained glycerol (1%), penicillin (189 mg), and M-9 salts in a total volume of 6 ml. Cells from the saline suspensions were added to the penicillin tubes to a concentration of 2.0 x 10^8 cells/ml and incubated for 5.0 hours. A dilution series (plated on glucose agar) provided the range to allow subsequent plating of the cells on glucose agar at about 100 cells/plate. The glucose plates were replicated onto glucose and glycerol agar. Colonies which were capable of growth on the glucose medium, but not on glycerol, were selected for further study.

2. Direct Plating Method

Plates were prepared which contained 0.5% glycerol, 0.02% glucose, and 5 µg/ml nitrosoguanidine in minimal medium solidified with 2% agar. Cells were grown up in nutrient broth, diluted in saline, and plated at about 100 cells/plate. Organisms which were incapable of growth on glycerol appeared as small colonies among the larger wild-type colonies.

G. Preparation of Phage Plate Stocks

The bacteriophage used in the preparation of plate stocks and for transduction was E-1°PA-1, and was obtained from R. R. Green. The media used for phage cultivation were prepared as described by Holloway, et al (23). One drop of log phase cells and 0.1 ml of a phage suspension containing about 5×10^6 phage/ml were combined in 2.5 ml of soft phage agar and poured over the surface of a plate containing 30 ml of phage bottom-layer agar. After 12 hours incubation at 37° C, the plates were soaked with 5 ml Pseudomonas phage broth for 30 minutes; the broth was pipetted from the plates and the cells removed by centrifugation. The supernatant was filtered through a Millipore (H.A. 0.45 µ pore size).

H. Transductions

Nutrient agar slants of the organisms were inoculated 15 hours prior to use. Each slant was flooded with 2 ml saline and 0.1 ml of the cell suspension was spread over the surface of a glycerol minimal agar plate. One-tenth ml of each phage suspension used was placed in a localized, labeled area on the plate and allowed to dry prior to

incubation at 37° C. Growth of transductants was counted after 48-72 hours.

I. Preparation of Cell-Free Extracts

Cells were grown in 800 ml of the appropriate medium (0.5% glucose or glycerol), collected by centrifugation during late log phase, washed with 0.85% NaCl, and frozen. The cells were thawed, resuspended in 20 ml 0.02 M potassium phosphate buffer, pH 7.0, and subjected to six to eight 10-second bursts of sonic oscillation with intermittent cooling in ice. Whole cells and cell debris were eliminated by centrifugation. All extracts were dialyzed for 4 hours against 4 liters of 0.02 M potassium phosphate buffer, pH 7.0, prior to use in enzyme analyses. In addition, each extract used in the determination of triose-phosphate isomerase activity was dialyzed against 0.02 M bicine, pH 8.5, using 200 ml volumes consecutively through 5 changes of buffer, each treatment of 30 minutes duration.

J. Preparation of Cells for Assay of L-α-glycerophosphate Dehydrogenase

Mutant organisms which were capable of growing in glucose but not in glycerol were prepared for assay of L-α-glycerophosphate dehydrogenase activity in whole-cell form. Endogenous substrates which could affect the reduction of MTT via other dehydrogenase enzymes in the cell were largely removed by treating the cells with distilled water at 0° C. Forty ml of 0.2% glucose were inoculated with 0.1 ml of a saline suspension of cells from a nutrient agar slant. The culture was removed from the shaker during late log phase and divided into two 20-ml portions. To one portion of cells (A), glycerol was

added to a concentration of 1%, and the culture was returned to the shaker. The remaining cells (B) were centrifuged, rinsed with distilled water, resuspended in 20 ml of distilled water, and placed in an ice bath for 4 hours prior to final centrifugation and freezing of the pellet. The glycerol-treated cells (A) were removed from the shaker after 4 hours, suspended in distilled water for 4 hours and treated in the manner described for "B".

K. Enzyme Assays

1. Glycerol Kinase

The phosphorylation of glycerol was measured by coupling with the L-α-glycerophosphate dehydrogenase reaction according to the procedure described by Lin, et al (16). The assay mixture contained: 0.3 ml of 0.1 M glycerol, 0.3 ml of 3 N hydrazine, 0.5 ml of 1 M sodium carbonate buffer at pH 9.5, 0.2 ml of 0.1 M ATP, 0.6 ml of 0.1 M $MgCl_2$, 0.2 ml of 0.02 M NAD, 0.3 mg L-α-glycerophosphate dehydrogenase, and cell-free extract in a final volume of 3.0 ml. The glycerol was omitted in the blank. NADH formation was measured at 340 mμ in a Cary Recording Spectrophotometer, Model 14 (Applied Physics Corporation).

2. L-α-glycerophosphate Dehydrogenase of Cell-Free Extract

L-α-glycerophosphate dehydrogenase was assayed according to Lin, et al (16) by measuring the rate of reduction of the tetrazolium dye, MTT (thiazolyl blue), to its formazan which absorbed maximally at 550 mμ. Measurement of dehydrogenase activity was enhanced by the addition of KCN which prevented the passage of electrons through the cytochrome system. The assay mixture contained: cell-free extract,

0.1 M phosphate buffer at pH 7.5, 0.1 ml of 1.0 M DL-glycerophosphate (α,β mixture), 0.1 ml of MTT (1 mg/ml), and 0.2 ml of 0.15 M KCN in a final volume of 3.1 ml. The substrate was omitted in the blank. The reaction was followed in a Cary Recording Spectrophotometer.

3. L-α-glycerophosphate Dehydrogenase of Whole-Cell Preparation

The differential rate of dye reduction by induced and non-induced preparations of whole cells on the addition of substrate was taken as a measure of L-α-glycerophosphate dehydrogenase activity. The frozen cells were thawed, suspended in 2.0 ml of 0.1 M phosphate buffer, pH 7.5, and 0.2 ml of the cell suspension was added to a solution containing: 1.6 ml of 0.1 M phosphate buffer, pH 7.5, 0.2 ml of 0.15 M KCN, and 0.1 ml of MTT (1 mg/ml). The reaction was followed for 6 minutes at 25° using a Coleman Junior Spectrophotometer at 550 mμ. At 6 minutes, 0.2 ml of 1.0 M DL-α-glycerophosphate was added to both the induced and non-induced preparations. The optical density was recorded at one minute intervals for another 8-10 minutes.

4. Triose-phosphate Isomerase

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

Spectrophotometer, by reversing the positions of cuvettes containing blank and sample.

CHAPTER III

EXPERIMENTAL RESULTS

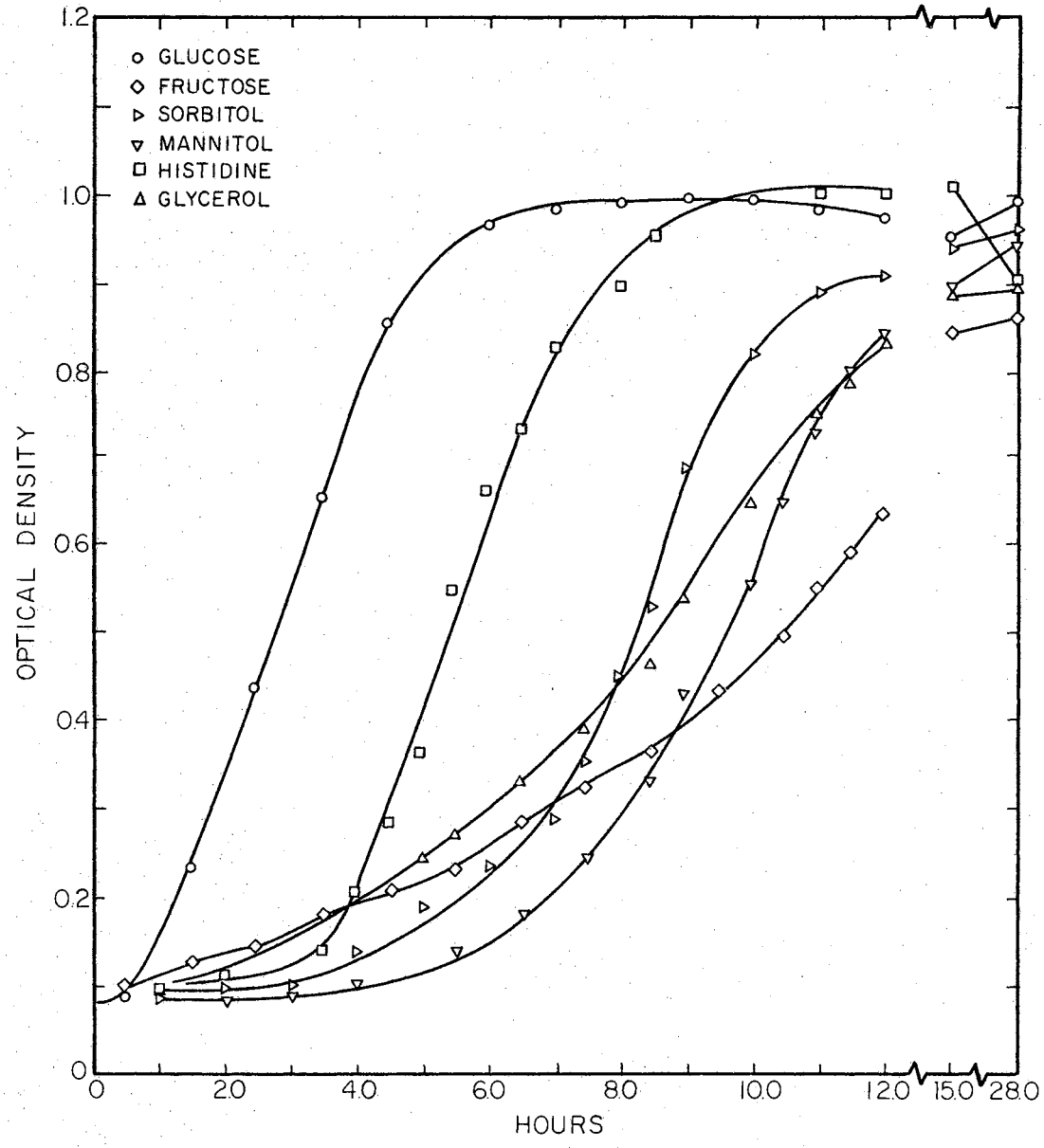
A. Control of Degradative Pathways in Pseudomonas aeruginosa

1. Inducibility of Degradative Pathways

Preliminary data were obtained demonstrating the ability of PA-1 to use as sole source of carbon and energy the following carbon sources: fructose, sorbitol, mannitol, histidine, glycerol, and glucose. To determine which of these catabolic pathways involved the production of inducible enzymes, glucose-grown log phase cells were inoculated into minimal media containing each of the compounds at a concentration of 0.5% and the growth of the cultures was recorded subsequently at 30 minute intervals. These growth curves are shown in Figure 2. The glucose medium was observed to allow an immediate resumption of growth. A lag in growth was observed for the other media tested. Fructose evidenced a very slow rate of growth initially, which increased significantly after several hours of incubation. Neither glycerol nor fructose supported growth at a rate comparable to that on glucose even after an apparent maximum growth rate was reached.

The inducibility of these pathways was further tested by growing PA-1 to log phase in each of the above media, resuspending half of each culture in fresh medium containing the same carbon source on

Figure 2. Growth of glucose-grown cells on glucose and other carbon sources.
Wild-type cells were grown to log phase in glucose minimal medium, harvested, and inoculated into minimal media containing various carbon sources, each at a concentration of 0.5%. Optical density was recorded at 30-minute intervals. (Not all readings are shown.)


GLUCOSE
FRUCTOSE
SORBITOL
MANNITOL
HISTIDINE
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10.0
12.0
15.0
28.0

which it was grown and the other half in glucose minimal medium. Growth resumed in all cases without the lag period observed when cells were transferred from a glucose medium. Growth in glucose was not delayed on transfer from the several different media. These data are shown in Figures 3, 4, and 5. As in the previous experiments using non-induced cells, growth rates on fructose and glycerol were quite slow compared to that on glucose even though fully-induced cells were used as inoculum.

2. Loss of Induced Enzymes

Cells were inoculated into minimal media containing 1.0% concentrations of fructose, sorbitol, mannitol, and histidine, and allowed to grow to stationary phase. Incubation of the cultures was continued for about two hours after maximum growth was reached. The cells were then transferred into fresh media of the same composition and also into glucose. In each case, growth was noted within one hour in glucose but a considerable lag period was noted for the other substrates. Induced enzymes for all four substrates appeared to be degraded within a fairly short time after the substrate was exhausted from the medium. These data are shown in Figures 6 and 7.

3. Repression by Glucose

The biosynthesis of inducible catabolic enzymes may be subject to repression by glucose. This mechanism is sometimes observed to produce a characteristic effect on the growth curve when the organism is incubated in a medium containing both the inducing substrate and glucose ("diauxie").

Figure 3. Growth of induced cells on glucose and on the inducing substrate.
Wild-type cells were grown to log phase in minimal medium containing a carbon source other than glucose at a concentration of 0.5%. Cells were harvested, inoculated into minimal medium containing the substrate on which they had been grown and into glucose minimal medium. Optical density was recorded at 30-minute intervals.

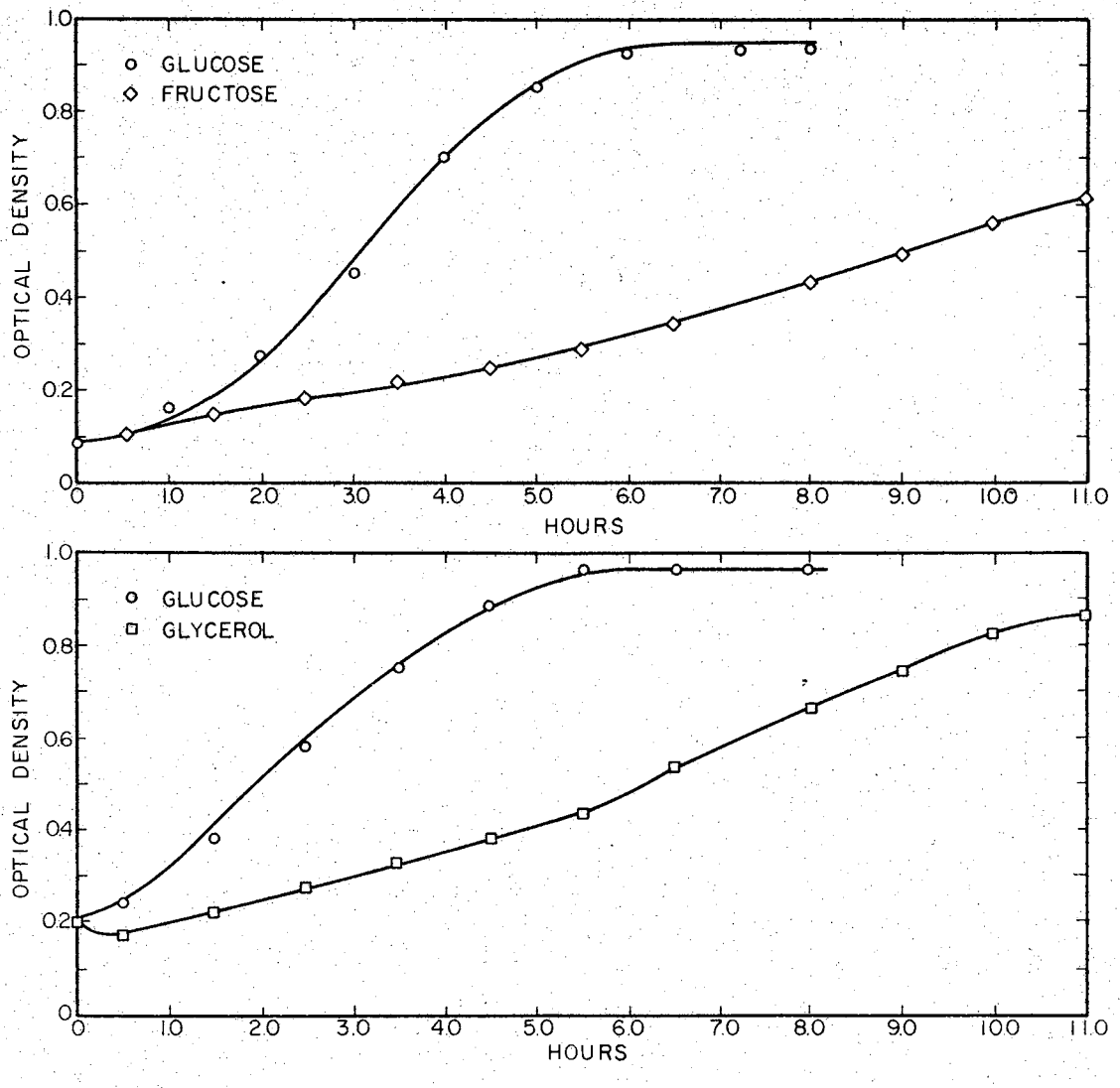
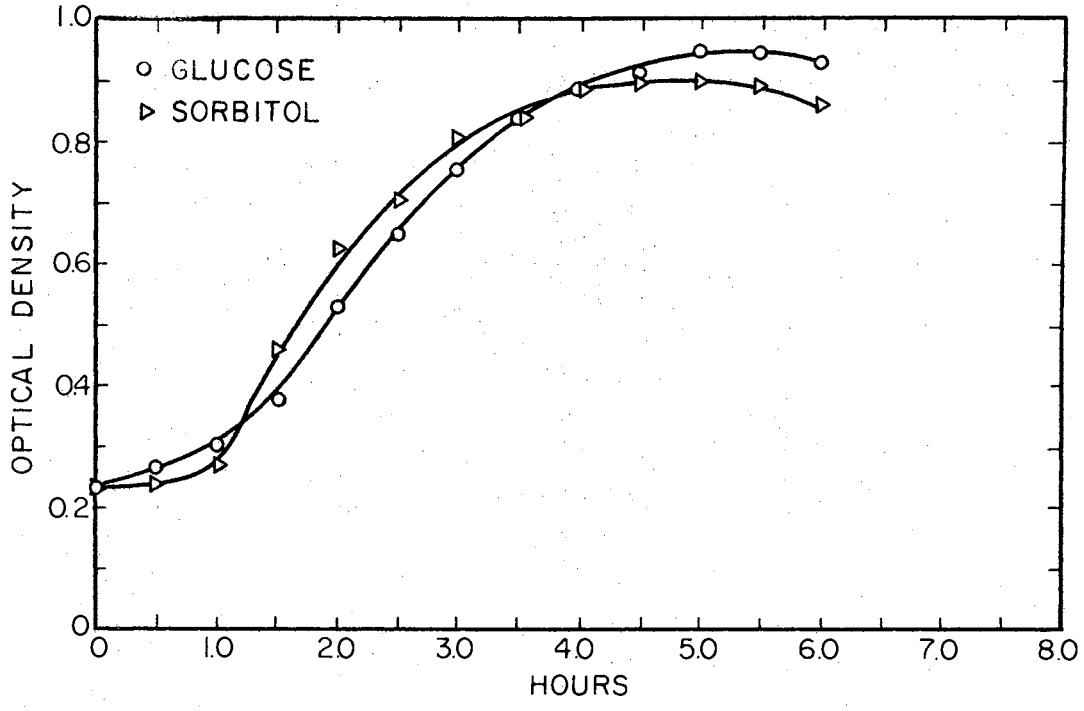

GLUCOSE
FRUCTOSE
OPTICAL DENSITY
HOURS
GLUCOSE
GLYCEROL
OPTICAL DENSITY
HOURS

Figure 4. Growth of induced cells on glucose and on the inducing substrate.
The experiment was performed as described for Figure 3 except that different carbon sources were used for growing cells.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SORBITOL
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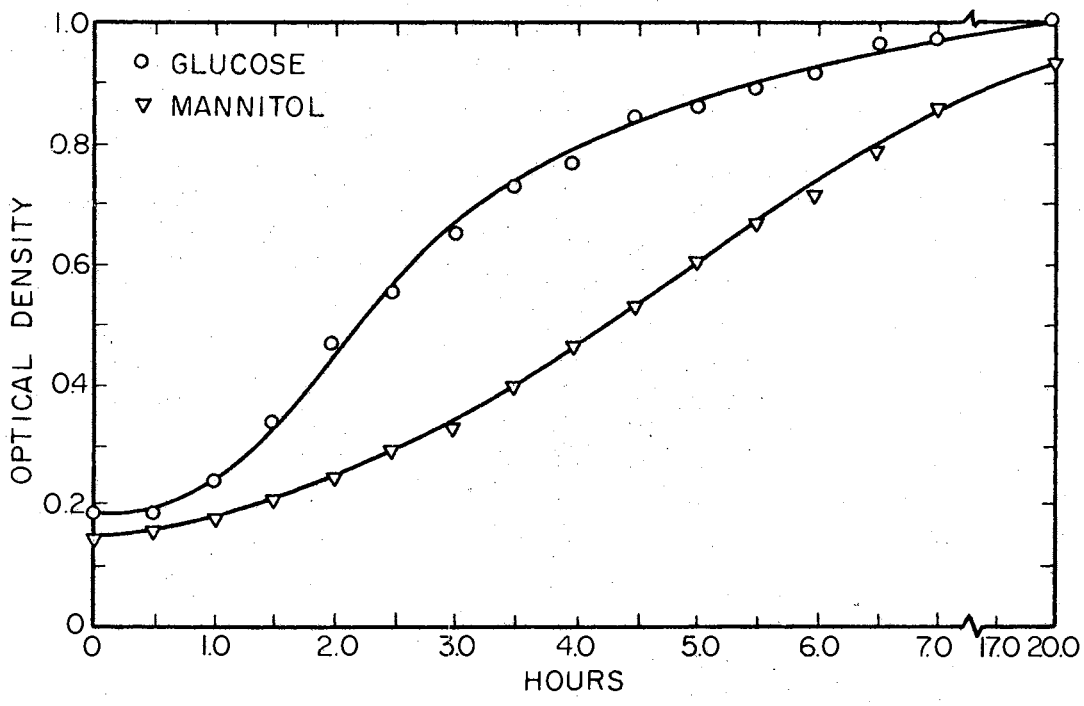
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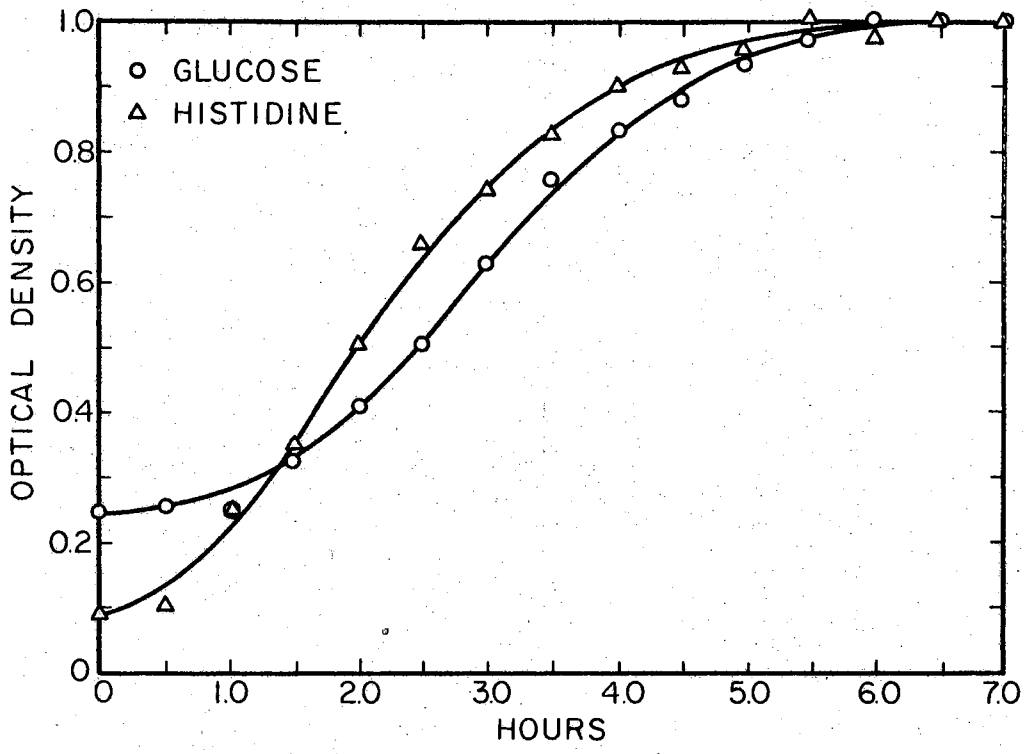
Figure 5. Growth of induced cells on glucose and on the inducing substrate.
The experiment was performed as described for Figure 3 except that histidine was used for growing cells.


1.0
0.8
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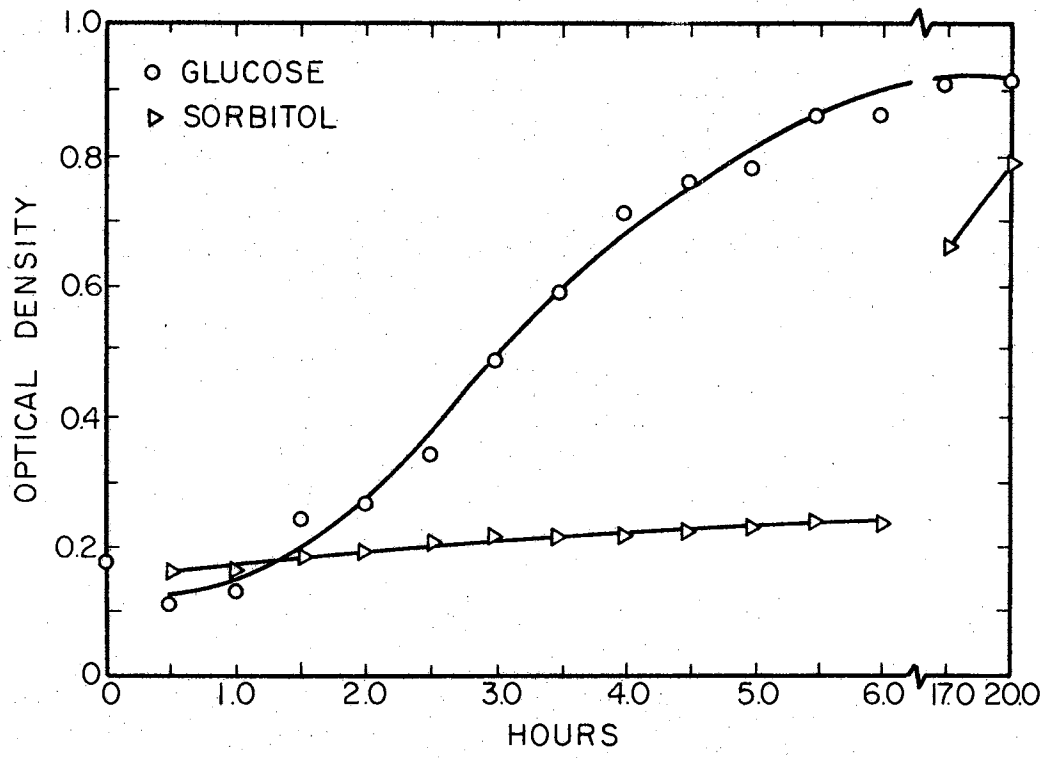
OPTICAL DENSITY
GLUCOSE
HISTIDINE
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
HOURS

Figure 6. Loss of induced enzymes in the absence of substrate.
Wild-type cells were grown in minimal medium containing 1.0% carbon source and incubation was continued for two hours after growth ceased. Cells were harvested and transferred to fresh medium of the same composition and also to glucose minimal medium. Optical density was recorded at 30 to 60 minute intervals.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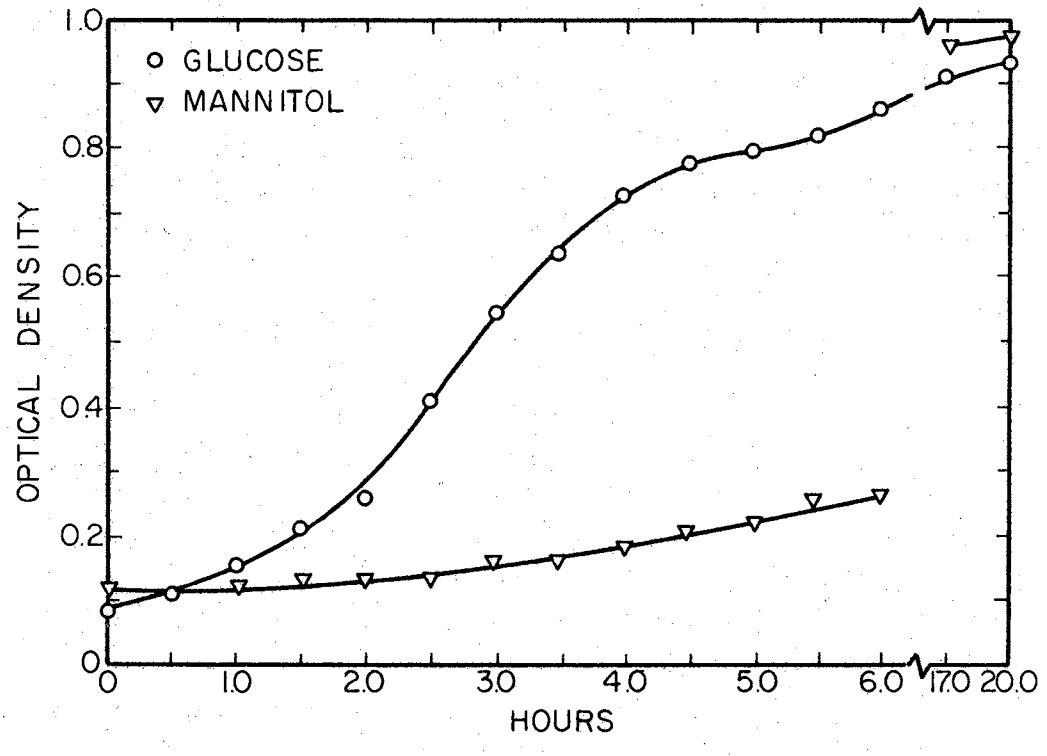
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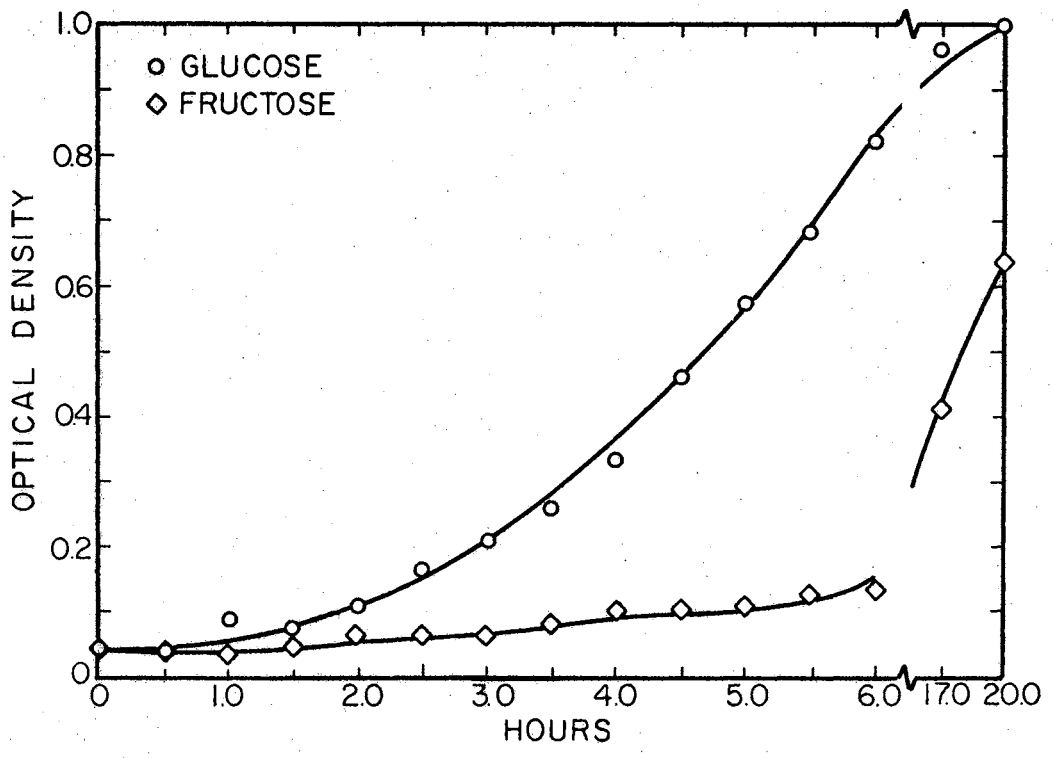
HOURS

Figure 7. Loss of induced enzymes in the absence of substrate.
The experiment was performed as described for Figure 6.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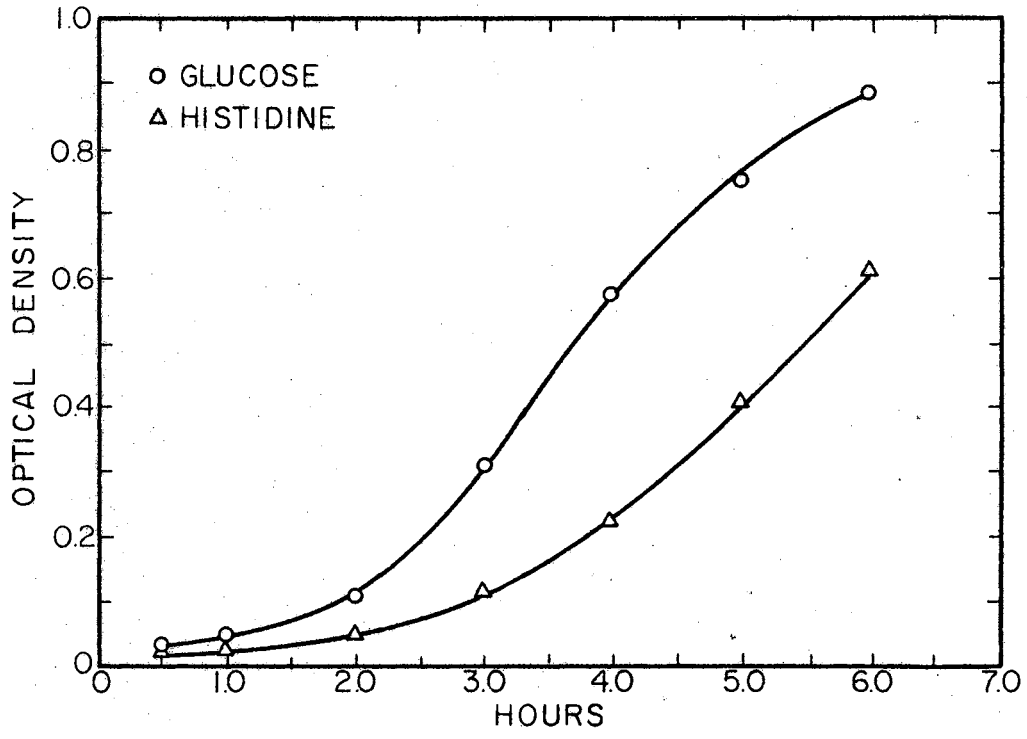
HOURS


GLUCOSE
HISTIDINE
OPTICAL DENSITY
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6.0
7.0
HOURS

If glucose is capable of repression, and assuming that glucose is used by the cells preferentially, then a "two-step" growth curve could result. On depletion of the glucose in the medium, a stationary period or lag would be evident prior to a resumption of growth on the second substrate. Growth curves of this type are often cited as a criterion for presence of the "glucose effect". Log phase cells, grown in glucose, were suspended in 0.1% glucose, and in mixtures of 0.1% glucose with: 1) 0.1% histidine, and 2) 0.1% glycerol. The optical density of each culture was recorded every 30 minutes. These data are shown in Figure 8. Neither mixture of substrates resulted in diauxic growth and this method, therefore, afforded no evidence of glucose repression.

Because repression by glucose may not be evidenced in growth data, analyses of substrate uptake during growth on glucose plus histidine and glucose plus glycerol were undertaken. Cells from the log growth phase in glucose were transferred to: 1) 0.1% glucose, 2) 0.1% glucose plus 0.1% histidine, 3) 0.1% glucose plus 0.1% glycerol. Aliquots were taken from the mixtures hourly and analyzed for the quantities of substrates present. Growth was measured as optical density every thirty minutes for all three flasks until stationary phase. These data are shown in Figures 9 and 10. The utilization of histidine and glucose was found to be concommitant. In contrast, the level of glycerol in the medium remained unchanged until the glucose was largely depleted. From these data, it can be concluded that glucose represses the utilization of glycerol but not that of histidine. Again, no diauxie was shown in growth on glucose plus glycerol.

Figure 8. Growth on glucose and on mixtures of substrates. Wild-type cells were harvested during the log phase from glucose minimal medium and resuspended in glucose medium and in medium containing glucose combined with a second carbon source. Optical density was recorded at frequent intervals. Experiment 1: 0.1% glucose (o); 0.1% glucose plus 0.1% glycerol (□). Experiment 2: 0.1% glucose (o); 0.1% glucose plus 0.1% histidine (△).

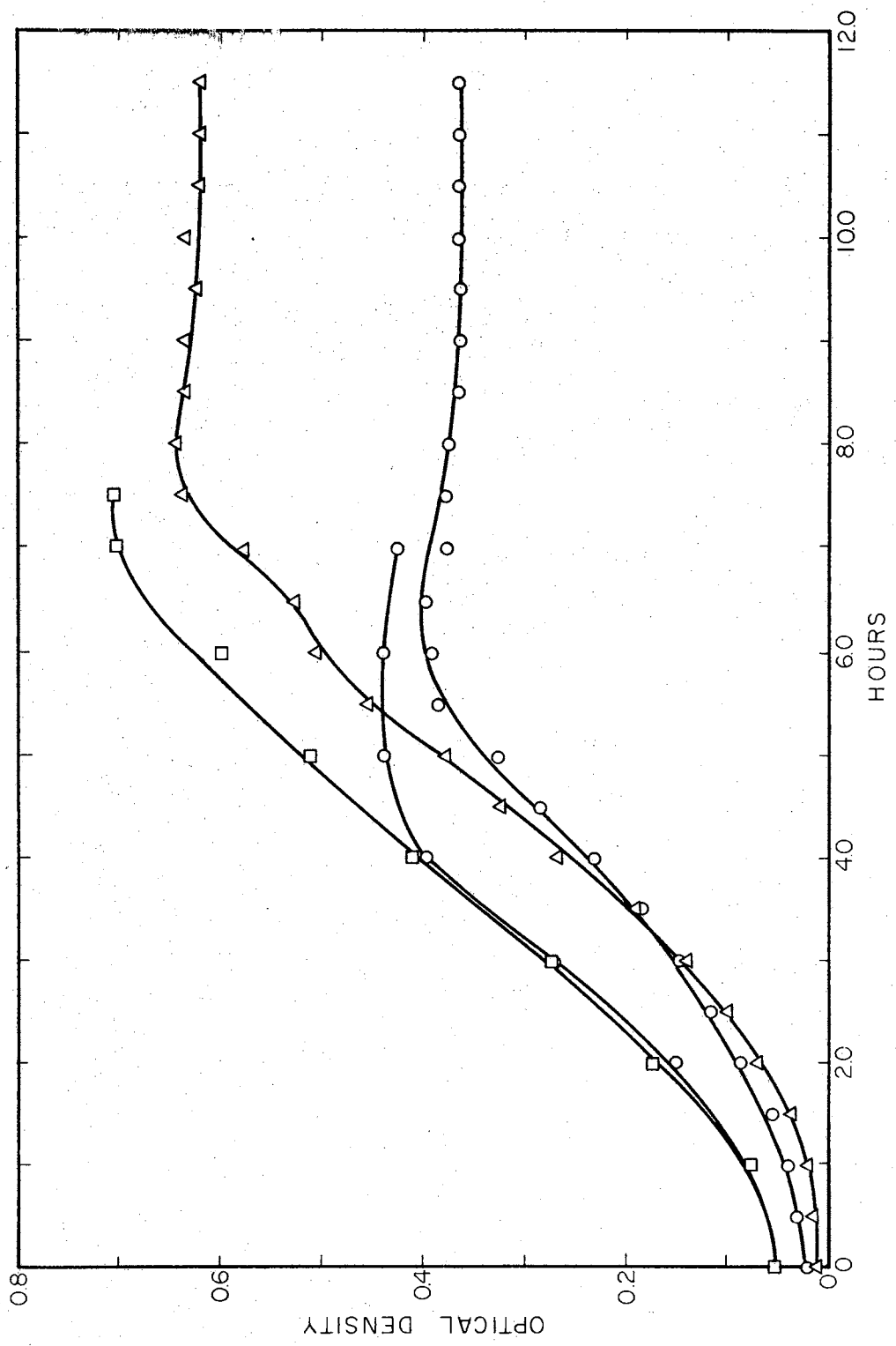

OPTICAL DENSITY
0.8
0.6
0.4
0.2
0
0
2.0
4.0
6.0
8.0
10.0
12.0
HOURS

Figure 9. Effect of glucose on utilization of histidine by glucose-grown cells.
Wild-type cells were harvested during the log phase from glucose minimal medium and inoculated into minimal media containing: (1) 0.1% glucose and (2) 0.1% glucose + 0.1% histidine. Optical density was recorded for both flasks hourly and samples were removed from the mixture and analyzed for glucose and histidine. Glucose concentration (●); histidine concentration (▲); optical density in glucose alone (○); optical density in glucose plus histidine (△).

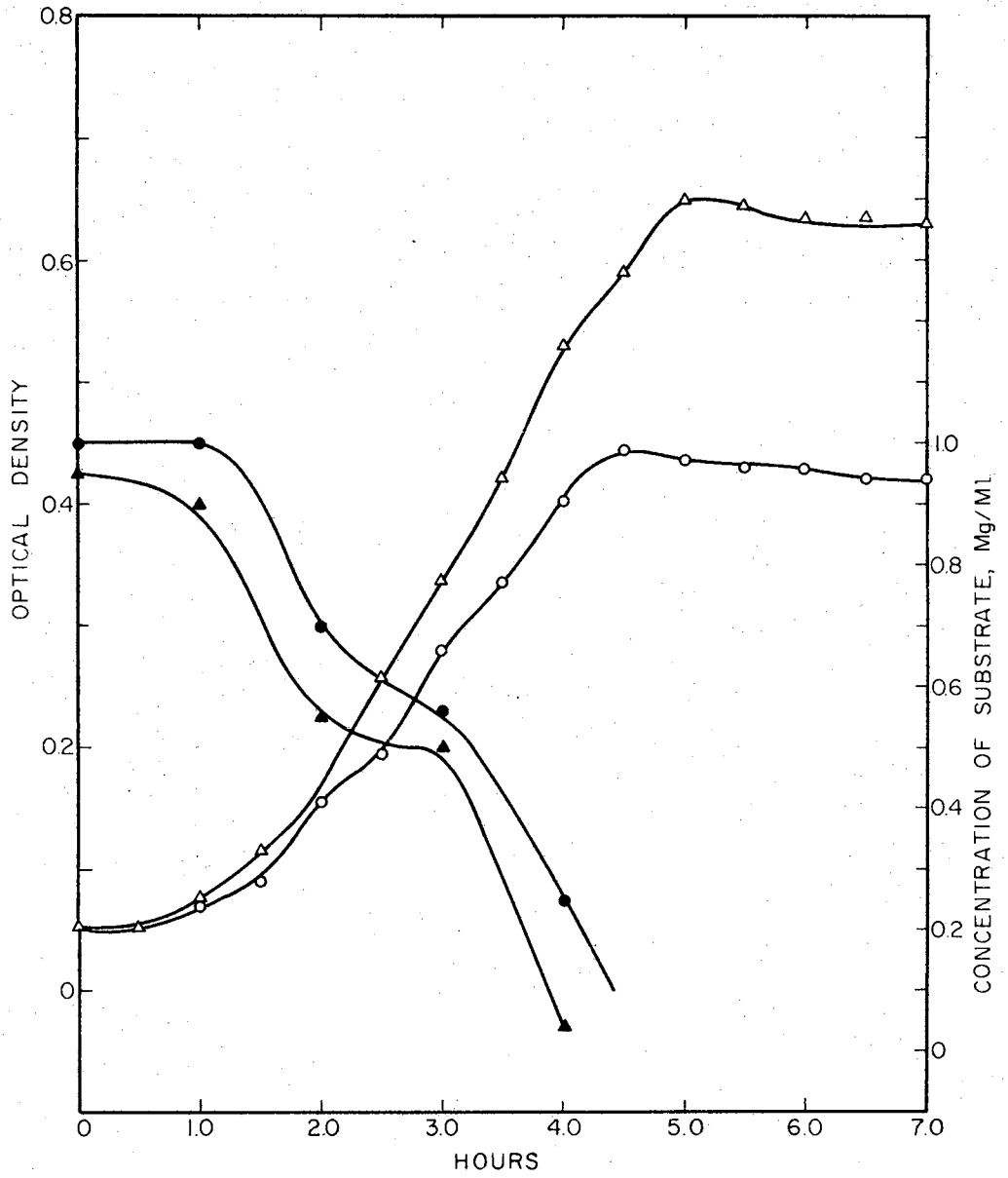
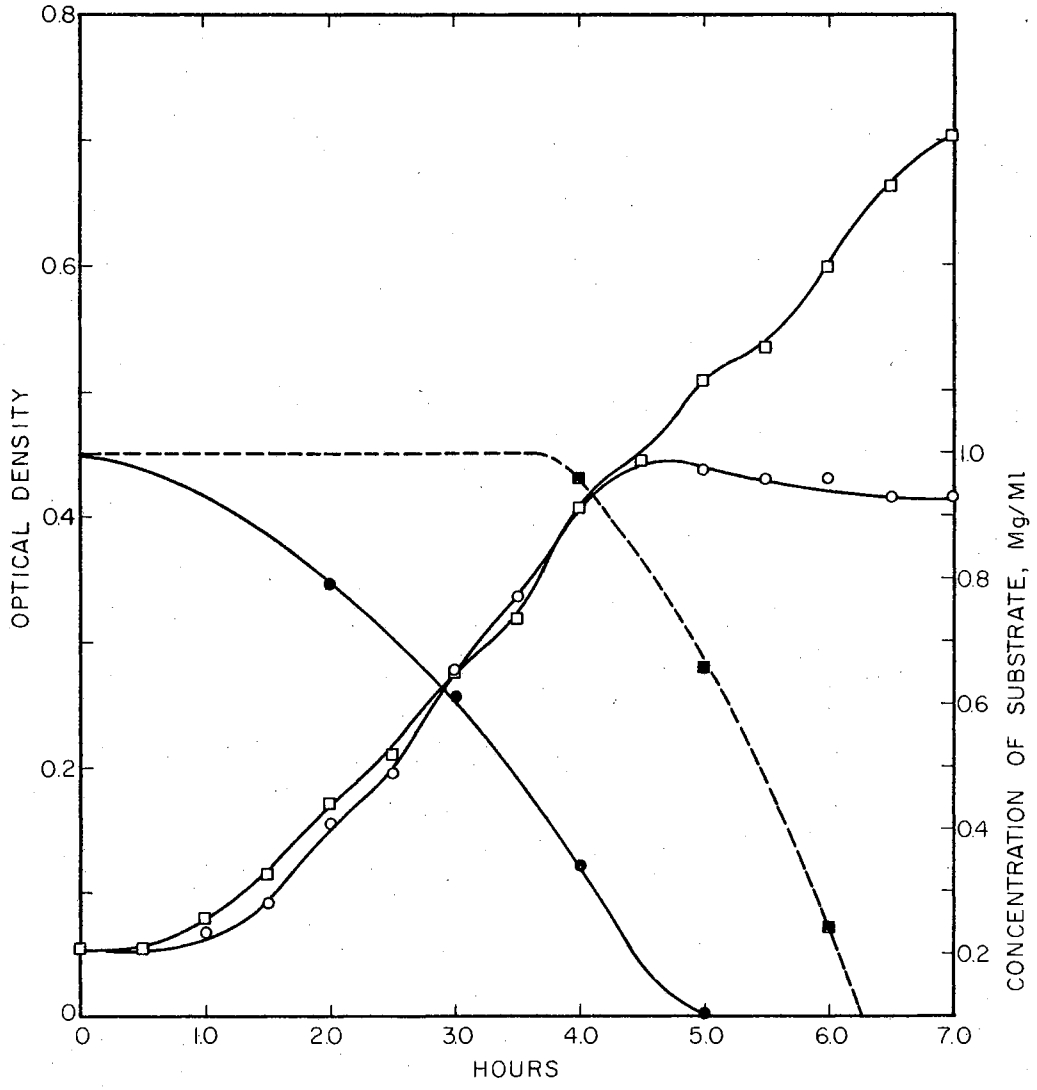

0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0
CONCENTRATION OF SUBSTRATE, Mg/Ml
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
HOURS

Figure 10. Effect of glucose on utilization of glycerol by glucose-grown cells.
The experiment was performed as described for histidine (Figure 9) except that the mixed substrate was composed of 0.1% glucose plus 0.1% glycerol. Glucose concentration (●); glycerol concentration (■); optical density in glucose alone (○); optical density in glucose plus glycerol (□).


OPTICAL DENSITY
0.8
0.6
0.4
0.2
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
HOURS
1.0
0.8
0.6
0.4
0.2
CONCENTRATION OF SUBSTRATE, Mg/Ml

4. Inhibition by Glucose

Because the degradation of glycerol had been shown to involve the production of inducible enzymes that were sensitive to repression by glucose, further investigations into the glycerol pathway were initiated. Although glucose was seen to repress the formation of the enzymes of the pathway, no evidence was available on the effect of glucose on the activity of pre-formed enzyme.

To test the response to glucose addition of cells fully induced to glycerol degradation, PA-1 was grown overnight from a small inoculum in glycerol minimal medium. The culture was diluted with fresh glycerol minimal medium and incubated to log phase. Cells were then harvested by centrifugation, resuspended in one-fourth the original volume of M-9 salts and used to inoculate six flasks with test tube side-arms. One flask contained 0.25% glucose medium (glucose control) and the other five contained 0.25% glycerol. Glucose was added to one flask of glycerol medium at zero time and to the remaining three flasks after one, two and three hours, respectively. The final concentration of glucose in all cases was 0.25%. The fifth flask containing glycerol was used as a control. All flasks were shaken at 37°, and at hourly intervals optical density was recorded and a sample removed from each flask for determination of substrate concentrations.

These data are shown in Figures 11 through 15. In Figure 11, which shows the two control cultures, it may be seen that glycerol-grown cells utilize glucose much more rapidly than glycerol. Glucose was depleted within 3 hours, while 11 hours were required for complete removal of glycerol. Figures 12 through 15 show the effect of

Figure 11. Growth and substrate utilization by glycerol-grown cells.
Wild-type cells were grown in glycerol minimal medium, diluted into fresh glycerol medium, harvested during log phase and used to inoculate five flasks of glycerol minimal medium (0.1% glycerol) and one flask of glucose minimal medium (0.1% glucose). Optical density was recorded for each flask hourly and samples were removed for determination of substrate concentration. Data for control flasks are shown in this figure and for flasks receiving substrate mixtures in Figures 12 through 15. Optical density in glucose (○); optical density in glycerol (□); glucose concentration (●); glycerol concentration (■).

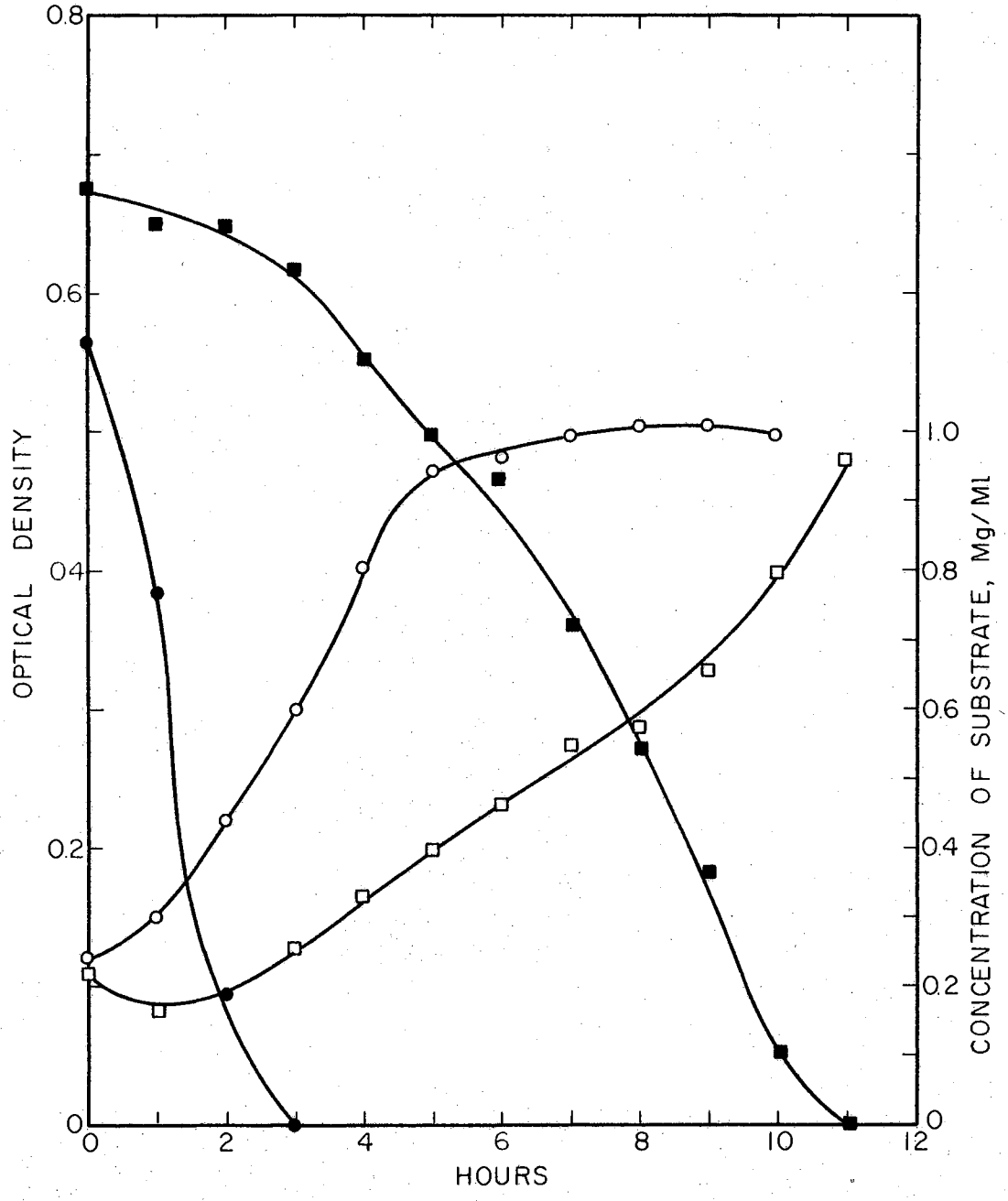

0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
0
2
4
6
8
10
12
HOURS
1.0
0.8
0.6
0.4
0.2
0
CONCENTRATION OF SUBSTRATE, Mg/Ml

Figure 12. Inhibition of glycerol utilization by glucose added at zero time.
Glucose was added at zero time to one flask of glycerol medium to a concentration of 0.1%. Preparation of flasks was described for Figure 11. Optical density (○); glucose concentration (●); glycerol concentration (□).

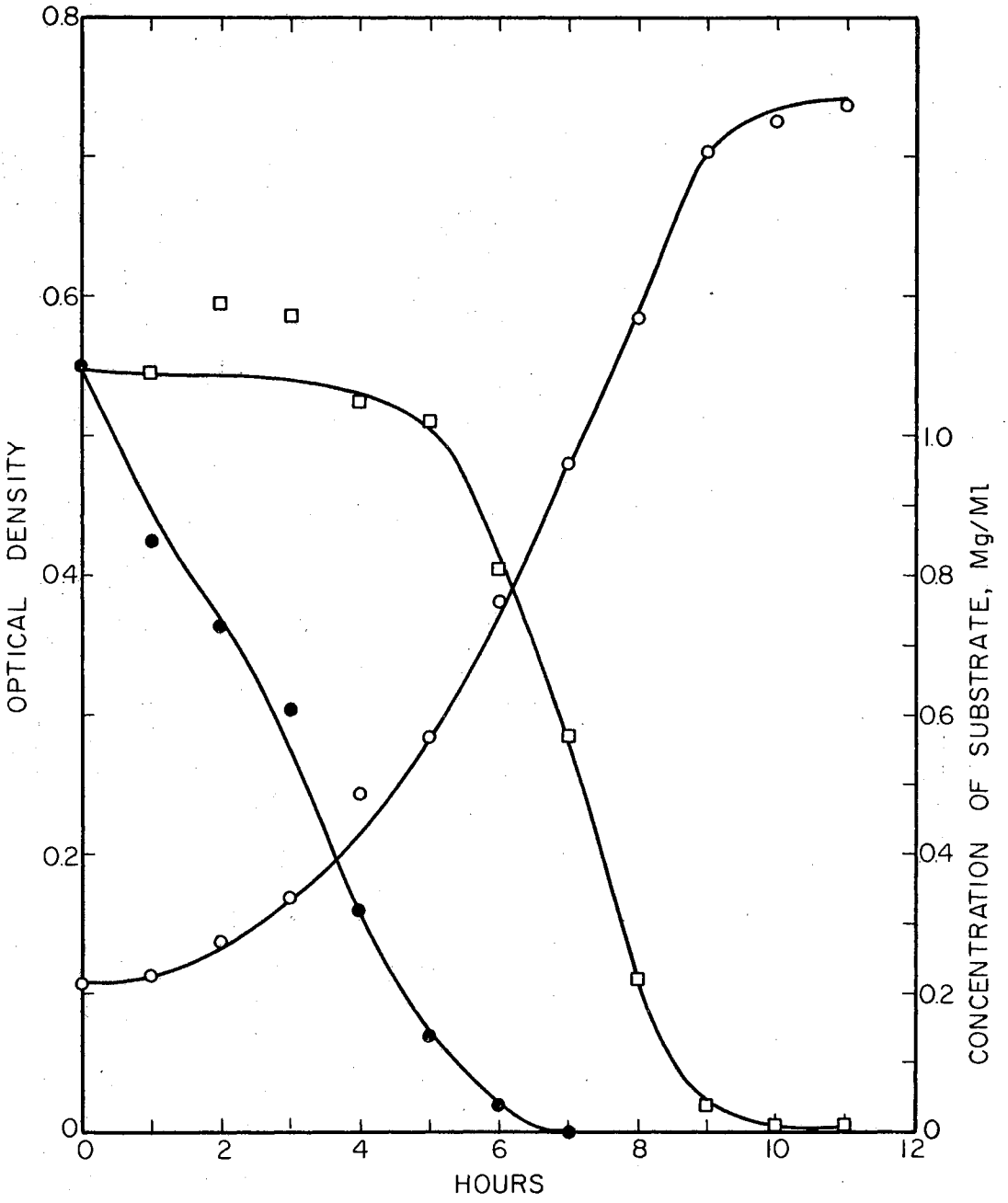

OPTICAL DENSITY
CONCENTRATION OF SUBSTRATE, Mg/Ml
HOURS
0
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
12

Figure 13. Inhibition of glycerol utilization by glucose added after one hour.
Glucose (0.1%) was added after one hour to a flask containing glycerol medium. Preparation of flasks was described for Figure 11. Optical density (○); glucose concentration (●); glycerol concentration (□).

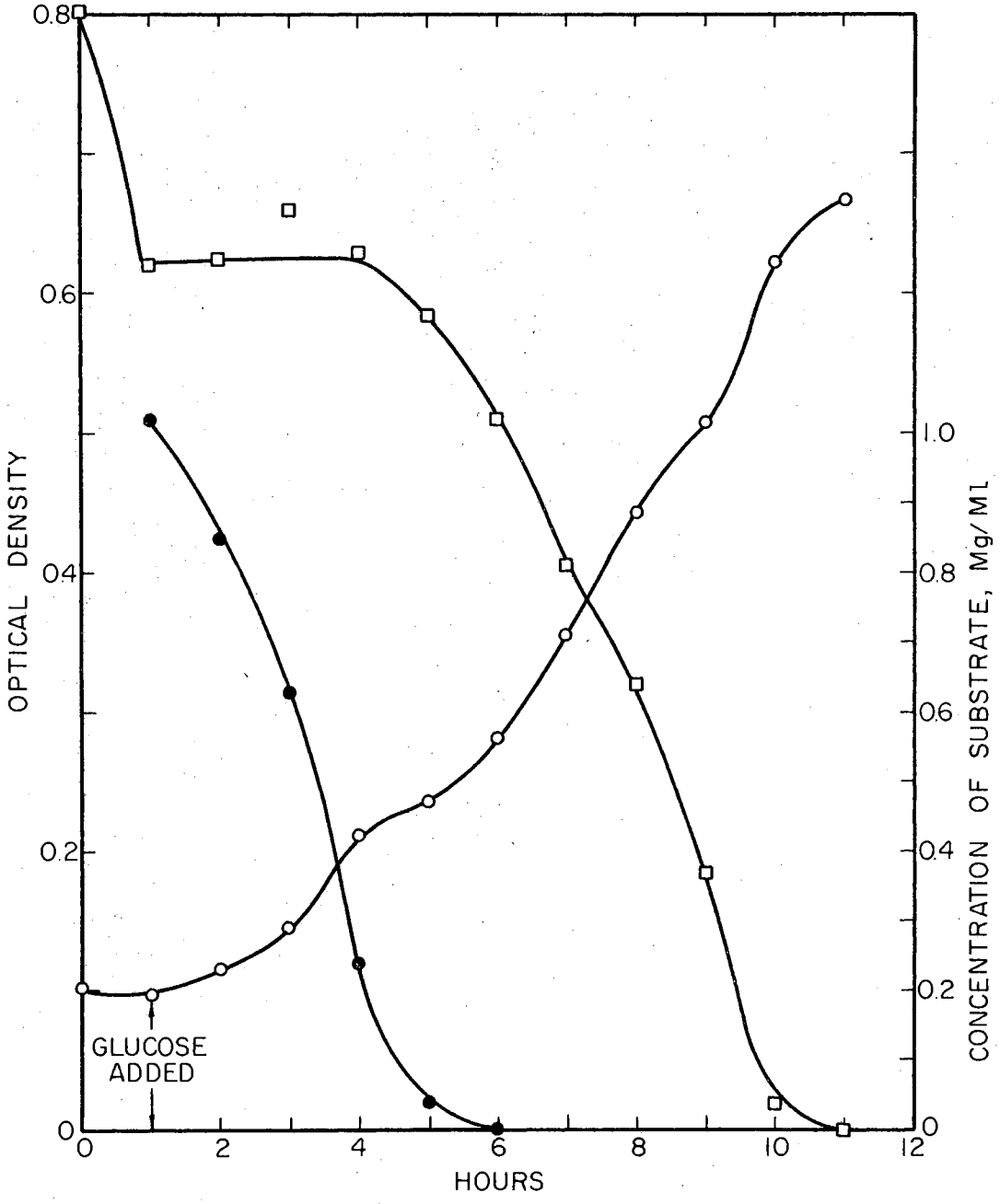

0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0
CONCENTRATION OF SUBSTRATE, Mg/Ml
GLUCOSE
ADDED
0
2
4
6
8
10
12
HOURS

Figure 14. Inhibition of glycerol utilization by glucose added after two hours.
Glucose (0.1%) was added after two hours to a flask containing glycerol medium. Preparation of flasks was described for Figure 11. Optical density (o); glucose concentration (●); glycerol concentration (□).

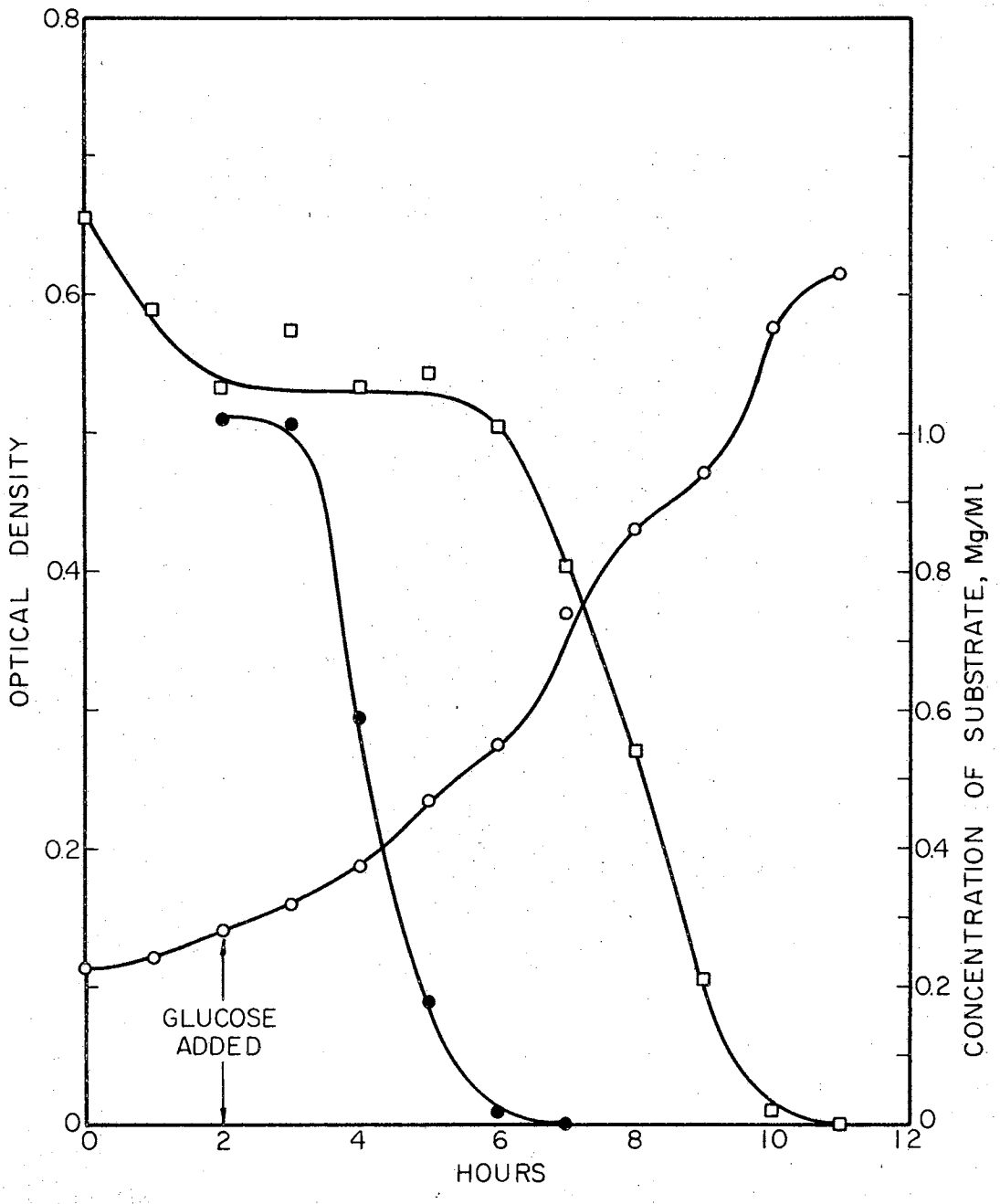
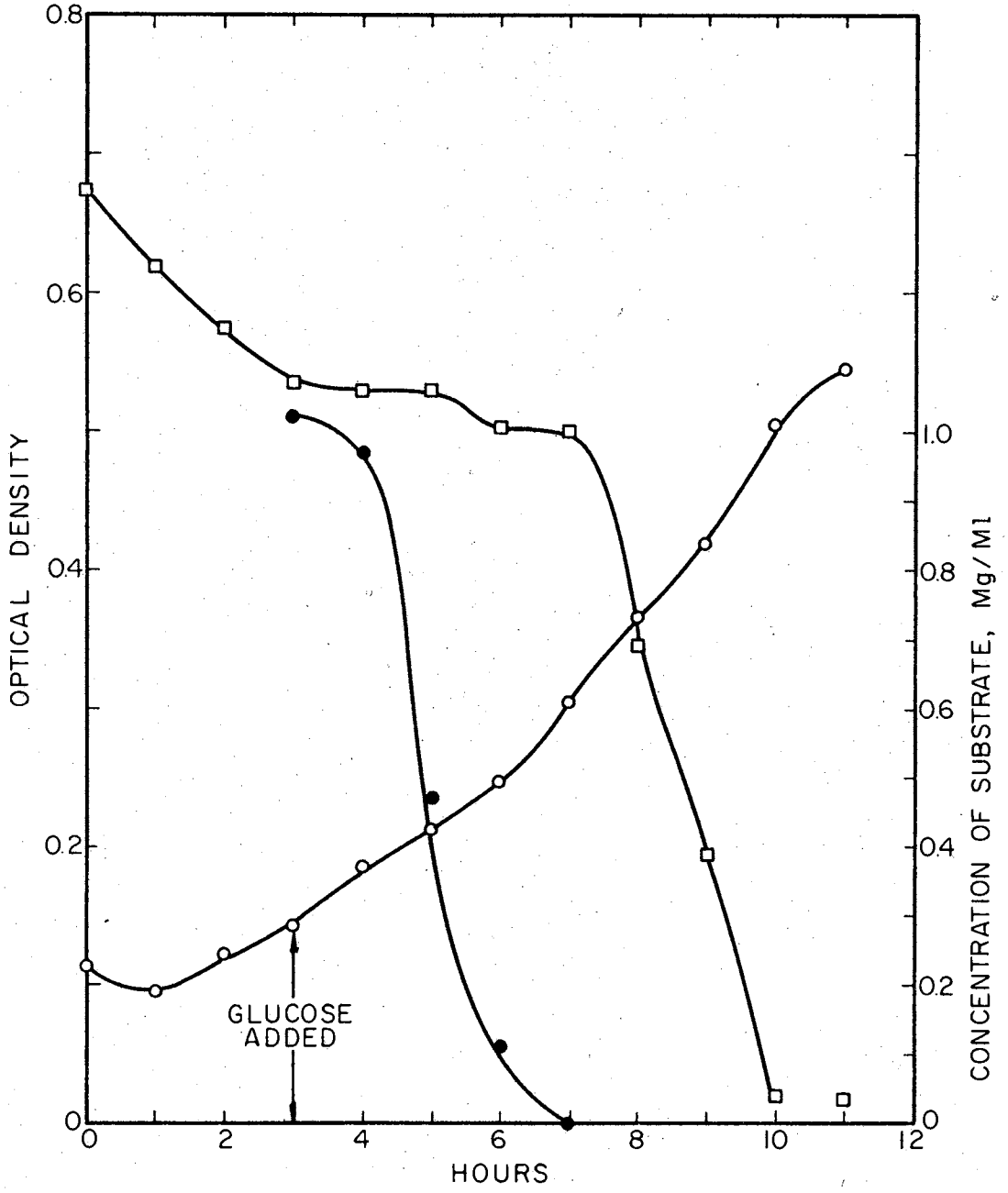

0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0
CONCENTRATION OF SUBSTRATE, Mg/Ml
GLUCOSE
ADDED
0
2
4
6
8
10
12
HOURS

Figure 15. Inhibition of glycerol utilization by glucose added after three hours.
Glucose (0.1%) was added after three hours to a flask containing glycerol medium. Preparation of flasks was described for Figure 11. Optical density (○); glucose concentration (●); glycerol concentration (□).


0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0
CONCENTRATION OF SUBSTRATE, Mg/Ml
GLUCOSE
ADDED
0
2
4
6
8
10
12
HOURS

addition of glucose to cells fully adapted to growth on glycerol. In each case, glycerol utilization was promptly suspended upon addition of glucose and did not resume until a basal low level of glucose had been reached. This experiment clearly shows that glucose inhibits glycerol utilization even though the required enzymes have been fully induced.

5. Induction of Histidine Degradative Enzymes by Urocanic Acid

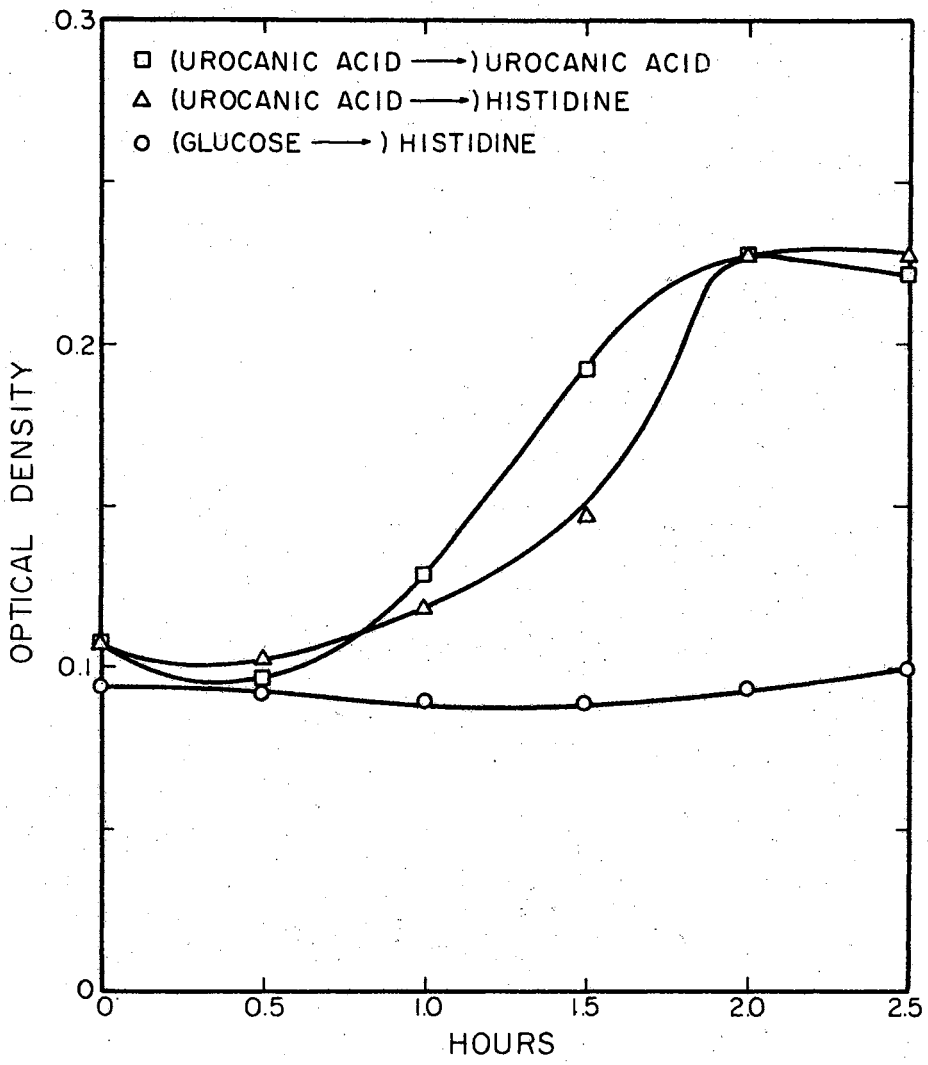
Urocanic acid has been shown to be the true inducer of the enzymes for histidine degradation in Aerobacter aerogenes (24). P. aeruginosa was tested for that characteristic by growing cells to log phase in urocanic acid, resuspending the cells in histidine and in urocanic acid media, and comparing the course of growth for these organisms with the growth of glucose-grown cells which had been resuspended in histidine. Growth curves are shown in Figure 16. No increase in cell density was observed for the non-induced culture during a 2.5 hour period of incubation. However, both the urocanate and histidine cultures which had been previously grown on urocanate resumed growth within one hour at an exponential rate. Therefore, it can be concluded that in P. aeruginosa, as in A. aerogenes, the enzymes for histidine degradation are induced by urocanic acid.

B. Glycerol Pathway in Pseudomonas aeruginosa

1. Glycerol Permease

A 100 ml culture of PA-1 in glycerol minimal medium was grown for 15 hours and the log phase cells were centrifuged, washed twice with a minimal salts solution, and suspended in 10 ml of minimal medium containing glycerol at approximately 3 mg/ml. Samples (0.5 ml) were

Figure 16. Induction of enzymes for histidine degradation by urocanic acid.
Wild-type cells were grown to log phase in minimal medium containing 0.5% urocanic acid, harvested, and inoculated into minimal media containing urocanic acid and histidine, respectively. Cells harvested from a log phase culture in glucose were simultaneously inoculated into histidine minimal medium. Optical density was recorded at 30-minute intervals.


□ (UROCANIC ACID ⟶) UROCANIC ACID
△ (UROCANIC ACID ⟶) HISTIDINE
○ (GLUCOSE ⟶) HISTIDINE
OPTICAL DENSITY
0.3
0.2
0.1
0
0
0.5
1.0
1.5
2.0
2.5
HOURS

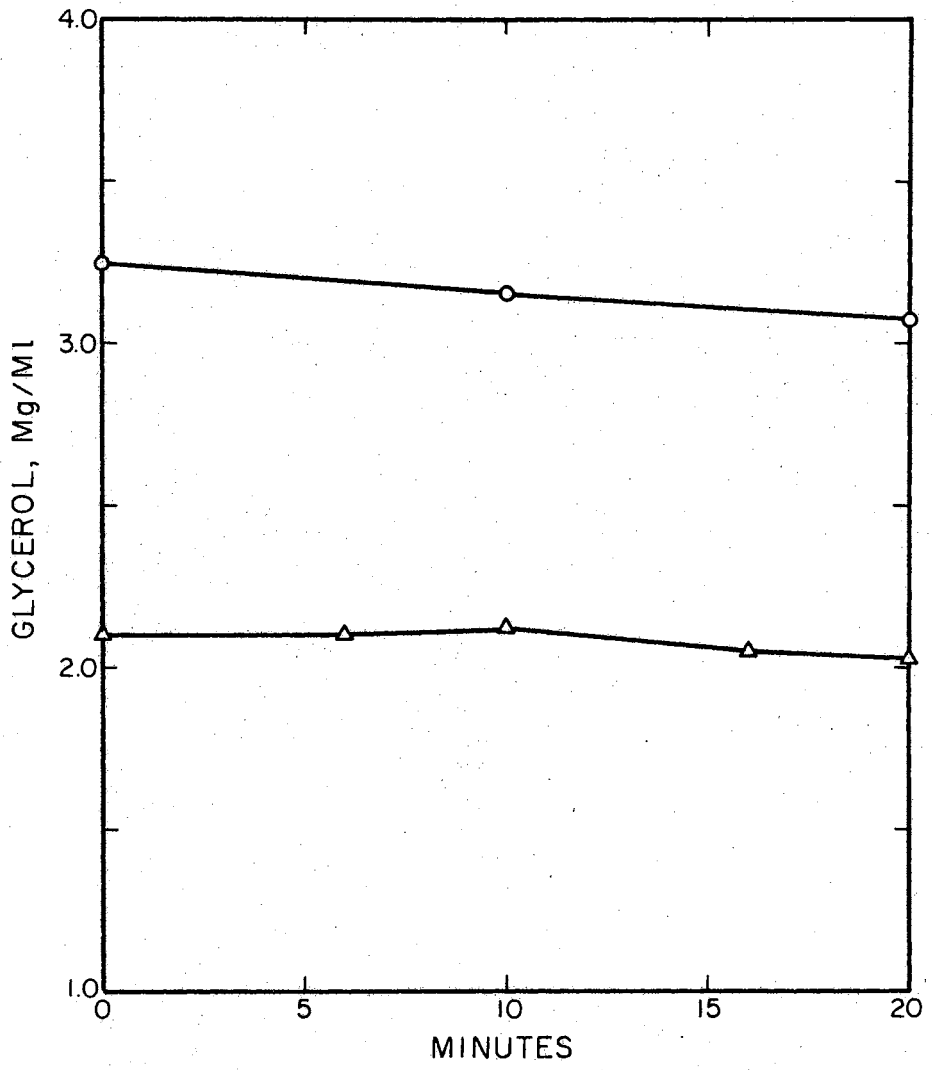
taken at zero, 10, and 20 minutes, and filtered immediately through a Millipore filter. The samples were frozen until the time of assay for glycerol content. The glycerol in the medium was found to have been reduced from an original concentration of 3.25 mg/ml to 3.10 mg/ml after 20 minutes.

In a second experiment using the same procedure, a 100 ml, 23-hour log phase culture was concentrated into a 10 ml volume containing glycerol at a slightly lower concentration of 2 mg/ml. As before, the glycerol in the medium was seen to decrease by about 0.1 mg/ml during the 20 minute period. Data for both experiments are shown in Figure 17. Since fully-induced cells were used in both experiments and essentially no uptake of glycerol occurred beyond that which might result from simple diffusion, it may be concluded that there is no mechanism for active concentration of glycerol in P. aeruginosa.

2. Growth on α-Glycerol Phosphate

Although the cell is generally considered to be impermeable to the passage of phosphorylated compounds, a specific transport mechanism for the uptake of α-glycerol phosphate has been reported for Escherichia coli (17). PA-1 was tested for ability to grow on this compound with negative results in all cases. Since α-glycerol phosphate was shown in later experiments, described below, to be a normal metabolite of glycerol in P. aeruginosa, the inability of the cells to use this compound for growth must be ascribed to lack of permeability.

Figure 17. Accumulation of glycerol by glycerol-grown cells. In Experiment 1 (○), a 15-hour log phase culture from glycerol minimal medium was harvested, washed, and resuspended in one-tenth the original volume of minimal medium containing glycerol at a concentration of 3.25 mg/ml. Samples were removed, filtered and assayed for glycerol content. Experiment 2 (▲) was similar except that a 23-hour log phase culture was used and the initial concentration of glycerol was 2.1 mg/ml.


4.0
3.0
2.0
1.0
GLYCEROL, Mg/Ml
0
5
10
15
20
MINUTES

3. Enzyme Assays

The enzymes of the glycerol degradative pathway in PA-1 (wild type) were assayed in extracts of cells which had been: 1) grown solely on glucose, 2) grown solely on glycerol, and 3) grown on glucose, then transferred to glycerol medium for a 4-hour period of induction. Each extract was assayed for glycerol kinase, α-glycerophosphate dehydrogenase, and triose phosphate isomerase activities. Enzyme activity levels for the three types of extract are shown in Table I.

Substitution of NAD for the tetrazolium dye in the dehydrogenase assay showed that no NAD-linked α-glycerophosphate dehydrogenase activity was present in either glucose- or glycerol-grown cells.

Based on these data, it may be concluded that the pathway for glycerol catabolism in P. aeruginosa involves glycerol kinase, a non-NAD-linked α-glycerophosphate dehydrogenase, and triose phosphate isomerase. This pathway is the same as that reported for E. coli.

Only the dehydrogenase was shown to be completely absent in non-induced cells. Growth on glycerol resulted in somewhat lower levels of kinase and dehydrogenase than were found in cells grown on glucose and induced by exposure to glycerol for four hours. Glycerol kinase was not completely absent in non-induced cells, but induction with glycerol increased the level of this enzyme almost three-fold. No evidence was obtained for induction of the triose phosphate isomerase by glycerol. The activity of this enzyme was considerably higher than those of the other two enzymes in all extracts, and the highest level found occurred in glucose-grown cells. Therefore, it would appear that this enzyme either is constitutive or is induced by growth on

TABLE I

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

Enzyme	Type of Cells		
	Glycerol-grown	Glycerol-induced	Glucose-grown
Kinase	0.013	0.023	0.008
Dehydrogenase	0.010	0.028	0.000
Isomerase	0.932	0.976	1.415

Assays were carried out as described in the text. Specific activities are expressed as change in optical density (MTT or NAD) per mg protein.

glucose.

C. Isolation and Characterization of Mutants

1. Isolation of Glycerol Mutants

a. Direct Plate Method

On plates containing 800 to 1000 colonies on glycerol-glucose-nitrosoguanidine agar, two distinct sizes of colony were noted. Numerous pinpoint-sized growths were observed among the larger wild type colonies; 183 of these small colonies were picked for further study. Each colony was transferred with an inoculating needle to nutrient agar, glucose minimal agar and glycerol minimal agar. Of the 183 colonies tested, 56 grew on neither glucose nor glycerol.

b. Nitrosoguanidine and Penicillin

Several unsuccessful attempts at isolation of glycerol mutants using nitrosoguanidine followed by penicillin selection prompted investigation of the effectiveness of penicillin as a selective agent and the optimal conditions for its use. One mutant organism which was capable of growth on glucose but not on glycerol had been isolated from prior sets of replica plates. A time study was run using this mutant organism and the wild-type (PA-1) to determine the relative mortality during treatment with penicillin. Both types of organism were grown up in nutrient broth, incubated for 4 hours in saline to deplete the metabolic pool, suspended at a concentration of 1.5×10^8 cells/ml in 6 ml of 1% glycerol containing 189 mg of penicillin, and incubated at 37° C with shaking. Samples were taken every 2 hours

and diluted for plate counts of viable cells. These data are shown in Figure 18. As a result of this experiment, an incubation time of five hours was chosen for the penicillin step in subsequent experiments. Using this technique, a total of 7 independent mutants were obtained from replica plates of nitrosoguanidine and penicillin-treated cells.

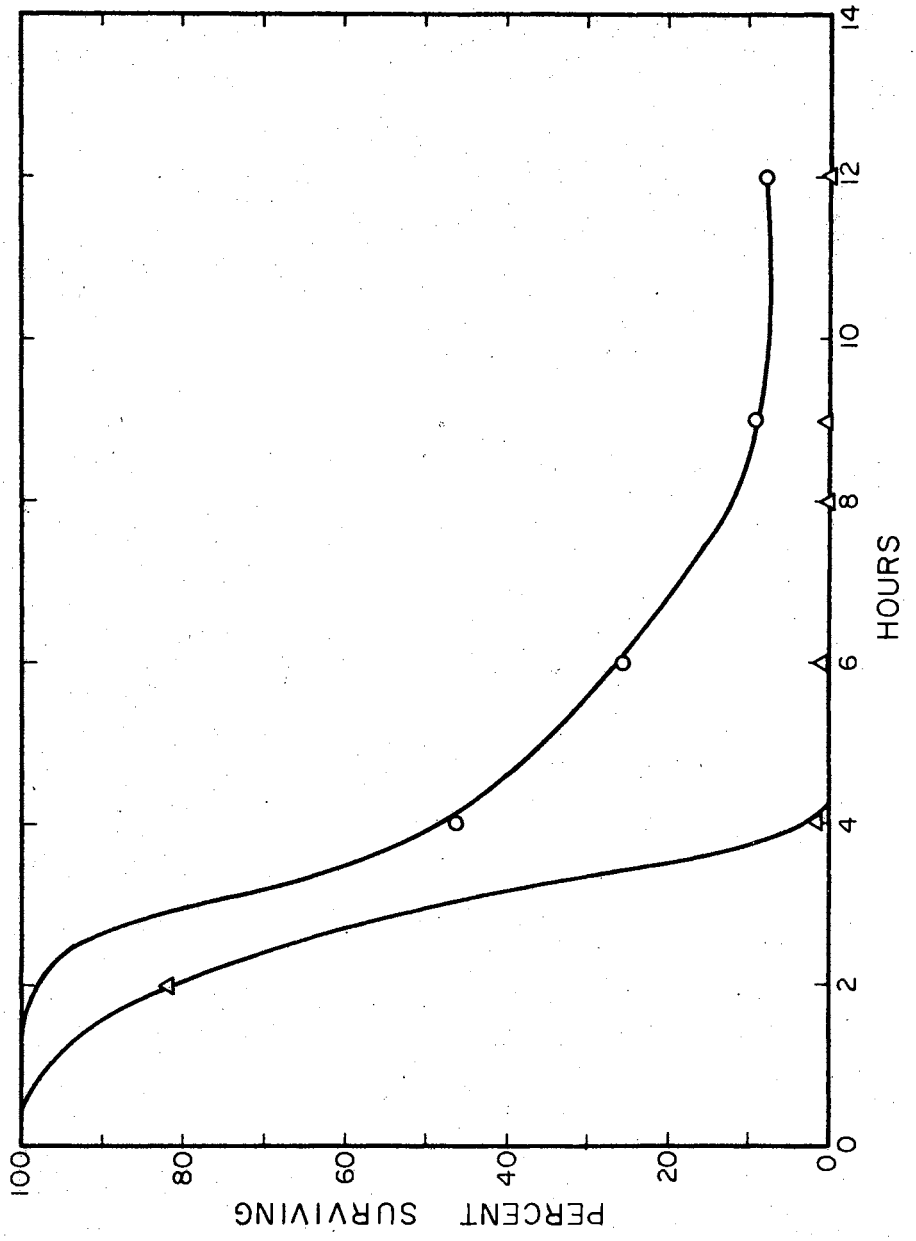
2. Characterization of Glycerol Mutants

a. Growth Studies

Mutants which had been isolated as very small colonies from nitrosoguanidine-containing plates (PA-1-623 through PA-1-678), were found to be unable to grow on either glucose, glycerol, or a medium containing both of these substrates. The addition of 0.02% yeast extract to the three media, however, revealed that the organisms were capable of growth on glucose to a level limited by the amount of yeast extract added. Yeast extract did not allow the utilization of the glycerol as carbon source. These mutants were further shown to require added yeast extract to grow on any of the carbon sources which had been tested originally with PA-1. In addition, the ability to grow on sorbitol and on mannitol was lost even with yeast extract present. PA-1 was checked for its ability to grow on the compounds tested at the beginning of the research and was found to be unable to use sorbitol, but growth on mannitol was the same as that previously observed. The loss of the ability to grow on both glycerol and on mannitol was therefore considered to be characteristic of these mutants. These mutants, all of which had identical growth patterns, were designated group II mutants.

Mutants which were obtained from replica plates (cells which had

Figure 18. Survival of mutant and wild-type cells in glycerol medium containing penicillin.
A glycerol mutant and the wild-type parent were grown in nutrient broth, aerated in 0.85% saline for 4 hours, suspended at a concentration of 1.5×10^8 cells/ml in 1% glycerol minimal medium containing 31.5 mg/ml of penicillin G, potassium, and incubated at 37°. Samples were removed from each culture at two-hour intervals for plate counts. Wild-type (Δ); mutant (O).


PERCENT SURVIVING
100
80
60
40
20
0
0
2
4
6
8
10
12
14
HOURS

been treated with both nitrosoguanidine and penicillin) grew readily on glucose but were unable to grow on glycerol. These mutants were designated group I mutants.

b. Enzyme Studies With Whole Cells

Four mutants from group I were tested for the presence of α-glycerophosphate dehydrogenase. Preparations of whole cells which had been specially treated as described before were used in the assays. One of these failed to show dehydrogenase activity by this method. The other three preparations showed marked increases in rate of dye reduction, on the addition of substrate, for the induced cells relative to the glucose-grown cells. The optical density readings for one mutant, C, are shown in Figure 19. Similar results were obtained with mutants E and 20 of group I. Mutant F had no dehydrogenase activity, as measured by this method.

c. Enzyme Studies With Cell Extracts

Extracts were prepared from one mutant of each group. Since all the mutants of group I had appreciable rates of reversion to wild type, only mutant E was used for enzyme studies. This mutant was chosen because its rate of reversion was the lowest of the group as judged by numbers of colonies obtained from glucose-grown cells plated on glycerol minimal agar. Only one mutant of group II was studied because it appeared that all these mutants possessed identical defects.

The enzyme activities measured in extracts of these two mutants are given in Table II. Neither of the extracts examined was found to

Figure 19. Measurement of α-glycerophosphate dehydrogenase activity in whole cells.
Mutant C (group II) was prepared as described in the text by growth on glucose, induction of one-half the cells with glycerol, washing and storage in ice-cold distilled water to remove endogenous substrates and freezing to increase permeability. Dehydrogenase activity was measured as reduction of MTT before and after addition of substrate, α-glycerophosphate. The non-induced portion of the culture, similarly treated, served as a control. The optical density of the control remained at 0.0 throughout the experiment.

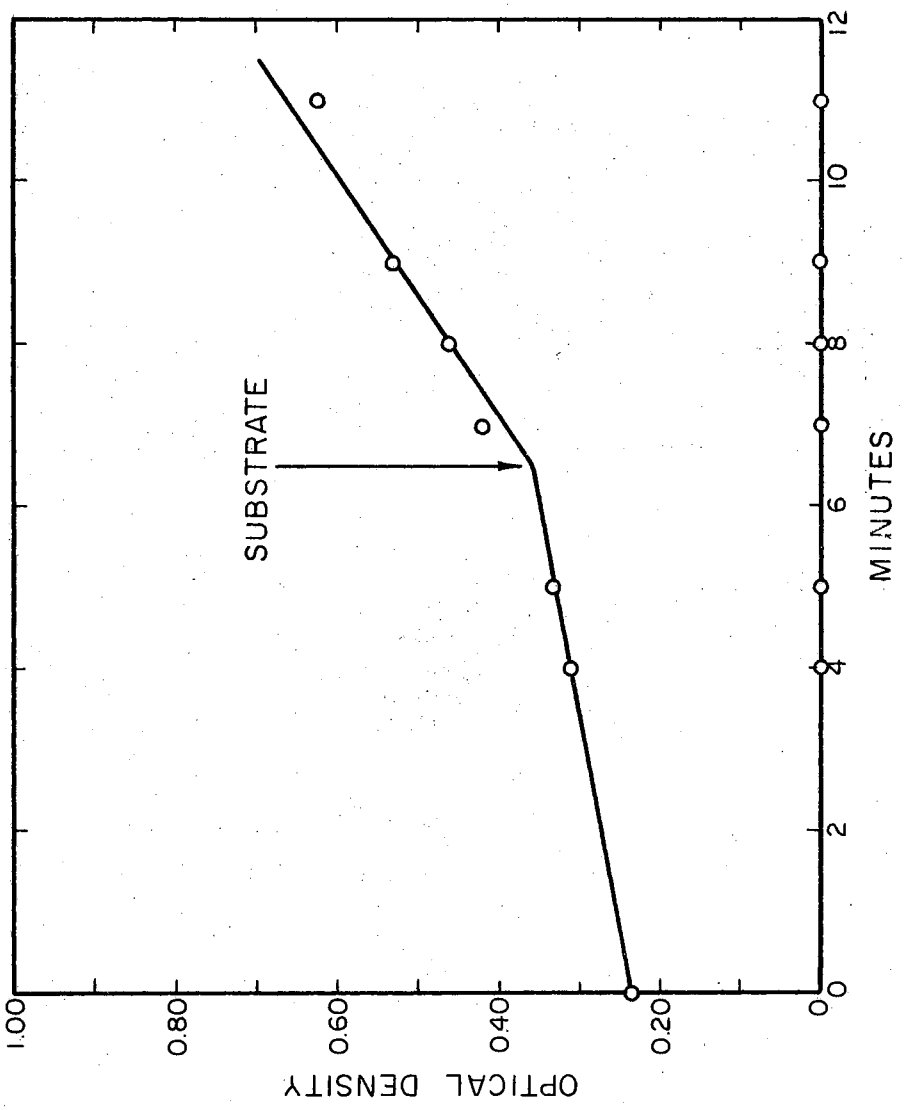

SUBSTRATE
OPTICAL DENSITY
1.00
0.80
0.60
0.40
0.20
0
0
2
4
6
8
10
12
MINUTES

TABLE II

SPECIFIC ACTIVITIES OF GLYCEROL ENZYMES IN CELL-FREE EXTRACTS OF MUTANT CELLS

Enzyme	Mutant	
	group I	group II
Kinase	0.015	0.025
Dehydrogenase	0.018	0.001
Isomerase	0.283	0.287

Cells of group I were grown in glucose minimal medium. Cells of group II were grown in glucose medium containing 0.02% yeast extract. Both groups of cells were induced for four hours in 1% glycerol. Specific activities are expressed as change in optical density (MTT or NAD) per minute per mg protein.

lack glycerol kinase activity. Both the extract that was prepared from a replica plate isolate (group I mutant) and the extract of a yeast extract-requiring organism (group II mutant) showed enzyme activities about equal to the wild type glycerol-grown or glycerol-induced extracts. Only the extract from the group II mutant was shown to lack appreciable α-glycerophosphate dehydrogenase activity. The activity of the other mutant extract was intermediate between that of the glycerol-grown and the induced wild type extracts. The extracts from both groups of mutants were shown to have a basal level of isomerase activity which was significantly lower than the enzyme activity observed for the wild type.

3. Transduction Studies

The organisms of group I yielded plate stocks with titers of 10^{10} to 10^{11} phage/ml. Attempts to prepare plate stocks of the organisms of group II were unsuccessful. Although all plates were prepared simultaneously, using the same media and phage suspension, no lysis occurred on plates containing any of five mutants from group II. Therefore, it was not possible to study transduction between the two groups of mutants.

Transduction studies were done between the members of group I, each mutant being treated with phage obtained from the other mutants of the group. The frequency of reversion to wild type growth was observed to be very high, making it impossible to determine whether transduction had occurred. The number of colonies observed within the area where phage had been applied was about equal to the number of revertants in other areas of the plates. Control plates using a

non-reverting auxotrophic mutant gave excellent results, indicating that the phage stocks used were capable of transduction.

D. Susceptibility to Phage

The initial attempt to prepare plate stocks of group II mutants showed that no lysis of cells occurred with treatment with E-1·PA-1. Twelve other strains of phage were tested for their ability to lyse two group II mutants and also a group I mutant. Eleven of the twelve phage were seen to lyse the group I organism, but none of these were observed to lyse either of the group II organisms.

The lack of susceptibility of the group II mutants to phage suggested a possible aberration of the cell wall structure which could be related to the use of glycerol as a biosynthetic component. Hauser and Karnovsky (25) have shown that glycerol is a precursor in the formation of rhamnose, and that fructose is an intermediate in that biosynthetic pathway. Furthermore, rhamnose has been found to be an integral component of the cell wall mucopeptide in Pseudomonas aeruginosa (26). Rhamnose and fructose were both checked to determine if the presence of these substrates in the growth medium could support growth of the mutants in the same manner as added yeast extract. The mutants were inoculated into:

1) 0.4% fructose
2) 0.2% fructose + 0.2% glycerol
3) 0.4% fructose + 0.02% yeast extract
4) 0.2% fructose + 0.2% glucose
5) 0.4% rhamnose
6) 0.2% rhamnose + 0.2% glycerol

7) 0.4% rhamnose + 0.02% yeast extract

8) 0.2% rhamnose + 0.2% glucose

Of these media, only the fructose plus yeast extract was observed to allow growth. The wild type (PA-1) was also shown to be unable to use rhamnose as carbon source. The possibility that rhamnose may be involved in the phenomenon has not been excluded, however, because no data are yet available concerning the permeability of the cells to that substrate. Preliminary data have been obtained for non-adsorption of phage to the mutants, revealing a relative resistance as compared to the wild type cell.

CHAPTER IV

DISCUSSION

In general, the data obtained in these studies support the conclusion that P. aeruginosa is subject to control mechanisms for catabolic pathways of the same types as those found in E. coli. Growth data obtained under three different conditions for several carbon sources indicate that pathways for these compounds are subject to genetic repression; i.e., the enzymes are not formed except in the presence of an inducer. The long induction periods required for growth of glucose-grown cells on other carbon sources (Figure 2), the immediate utilization of these same compounds by induced cells (Figures 3, 4 and 5) and the loss of induced enzymes after exhaustion of substrate (Figures 6 and 7) are evidence for genetic repression. Failure of glucose-grown cells to utilize glycerol in the presence of glucose is presumptive evidence for catabolite repression, or the "glucose effect". A newer control mechanism, inhibition of glycerol utilization by glucose in cells fully-induced by glycerol, was also demonstrated.

The two phenomena, repression by glucose and inhibition by glucose, are difficult to distinguish experimentally. Both mechanisms are seen to prevent the normal utilization of specific substrates in the presence of glucose. The differentiation of the two mechanisms has been somewhat complicated recently by a report that diauxic growth

of *E. coli* on glucose and lactose is caused by interference of glucose with the uptake of lactose, thus reducing the internal concentration of inducer (27).

The data for uptake of histidine from a glucose-histidine mixture clearly show that neither mechanism affects histidine catabolism in PA-1. In contrast, Lessie and Neidhardt (28) have found the histidine pathway of another strain of the same organism to be quite sensitive to repression by glucose or by succinate.

Glucose was effective in preventing the dissimilation of glycerol by *P. aeruginosa*, strain 1, in all cases. The abrupt halt in uptake of glycerol by induced cells on the addition of glucose possibly implies that the enzyme, glycerol kinase, had been rendered inactive. Glycerol was shown not to be actively transported into the cell at a rate greater than that of free diffusion. The rate of uptake of glycerol prior to addition of glucose, therefore, probably represents the rate of one-way diffusion of glycerol into the cells where phosphorylation by glycerol kinase prevents its exit. The uptake of glycerol would continue at a constant rate per cell in the presence of the active kinase. Very low levels of glucose were seen to prevent the use of glycerol. If competitive uptake of the substrates were involved to a significant degree, some evidence of increased glycerol uptake should have become increasingly apparent at these low levels of glucose.

The uptake data which were obtained for non-induced cells growing in a mixture of glucose and glycerol are typical of the results which have been cited as evidence of glucose repression by many different investigators. Some of these investigators have also corroborated their uptake data by specific enzyme analyses, demonstrating the

absence of the repressed enzyme during the metabolism of glucose. Such assays were not carried out in the present study.

Since it does not appear that in P. aeruginosa, strain 1, a permease is involved in glycerol accumulation, prevention of induction by a hindrance of glycerol accumulation, per se, appears unlikely. In view of the other data obtained, another explanation for the "glucose effect" can be advanced. The glycerol enzymes (or at least one required step in glycerol utilization) are clearly subject to inhibition by glucose. This inhibitory effect of glucose would prevent the detection of any induced enzymes of the glycerol pathway until the glucose had been depleted. The possibility exists that induction by glycerol is not prevented by glucose; instead, the enzymes are synthesized normally but are rendered inactive until the metabolism of glucose ceases. Considerable further study will be required to clarify the exact mechanism involved.

The absence of a discreet lag period between the time at which glucose is depleted from the medium and the observed uptake of glycerol indicates that the induction of the glycerol enzymes must be very rapid if true repression of enzyme synthesis had occurred during glucose metabolism.

The pathway of glycerol dissimilation in P. aeruginosa, strain 1, was found to involve the same intermediates as in aerobically-grown A. aerogenes strain 1041 or 1033 and E. coli strain K10. Both of these organisms differ, however, in certain aspects of their treatment of glycerol from the strain of P. aeruginosa used in this study. The NAD-linked pathway of anaerobically-grown A. aerogenes strain 1033 may be considered absent in PA-1 as in E. coli and A. aerogenes

strain 1041, since no reduction of NAD was observed in fully induced cell-extracts. The formation of dihydroxyacetone by an NAD-linked glycerol dehydrogenase and the participation of NAD in the oxidation of α-glycerophosphate were excluded as possible routes of metabolism by testing the cell extracts for their capacity to reduce NAD on the addition of α-glycerophosphate or glycerol as substrate.

E. coli differs from PA-1 in its ability to transport α-glycerophosphate into the cell. Both organisms are impermeable to α-glycerophosphate by free diffusion; therefore, PA-1 may be considered to trap glycerol on its diffusion into the cell in the same manner as has been noted for E. coli. In E. coli, the glycerophosphate permease and dehydrogenase, and glycerol kinase are induced by growth on either α-glycerophosphate or glycerol. In mutant cells which lacked the glycerol kinase, however, glycerol was ineffective as an inducer, but α-glycerophosphate induced both the permease and the dehydrogenase (11). Since no mutants were obtained during the present research which lacked glycerol kinase activity, it is not possible to speculate as to whether the true inducer for the pathway is glycerol, or whether the enzymes are induced by α-glycerophosphate as in E. coli. The lack of a transport system for α-glycerophosphate would seem to preclude any direct advantage to the cell of such a mechanism of induction.

The isomerase data obtained for the wild-type cell extracts show little significant difference between the induced and non-induced cells. Constitutive synthesis of the isomerase could account for this condition. Alternatively, the high levels of enzyme formed during both conditions of growth could be explained by induction by glucose and by glycerol. The latter possibility cannot be excluded since no

data are available for this enzyme during growth on substrates which are not degraded by the glycolytic route. Furthermore, the data do not preclude the possibility that two isomerase enzymes are present in this organism, one enzyme, formed during growth on glucose, mediating the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate and a second enzyme mediating the reverse of that reaction, which is specifically inducible by glycerol. Although the isomerase reaction is normally considered to be reversibly mediated by a single enzyme, the peculiar pathway for glucose degradation in Pseudomonads gives the isomerase step two distinct functions, one anabolic and another catabolic, depending on the substrate being metabolized. Separate enzymes are synthesized for other reactions of intermediary metabolism which function in both synthesis and catabolism (29). Therefore, teleonomic reasons may be seen which could give credence to the possibility that special control mechanisms may have evolved for this organism. Breakdown of glucose via the Entner-Doudoroff pathway does not involve the formation of dihydroxyacetone phosphate; instead, glyceraldehyde phosphate and pyruvate are formed directly from cleavage of a six-carbon precursor (Figure 1). During growth on glucose, therefore, biosynthesis of triglyceride and any other derivative of glycerophosphate would be dependent on the formation of dihydroxyacetone phosphate and its subsequent conversion to glycerophosphate. Because glycerophosphate is readily available to the cell during catabolism of glycerol, the isomerase step would have the single function of shuttling dihydroxyacetone phosphate into the mainstream of glycolysis.

Dihydroxyacetone phosphate was not specifically tested as a

substrate with cell extracts. However, the assay method used required that dihydroxyacetone phosphate be produced from glyceraldehyde-3-phosphate by an isomerase present in the extract. If two distinct isomerases are formed by the cell, the enzyme measured need not have been induced by glycerol, since the highest levels were observed in glucose-grown extracts. This explanation appears improbable, however, since cells grown on glycerol possessed high levels of isomerase when measured in the same way.

The low level of isomerase activity for both extracts prepared from mutants deserves particular attention. If the isomerase is constitutive or is inducible by either glucose or glycerol, the low activity could be due to production of an altered enzyme molecule. If there are two isomerases, one inducible by glucose and the other by glycerol, the activities measured in the mutant extracts may represent residual glucose-induced enzyme which had been formed during growth of the cells on glucose prior to a four-hour period of induction by glycerol. In the latter case, the glycerol-induced isomerase could be completely absent and this could explain the inability of the two mutants to grow on glycerol. If a single constitutive, reversible isomerase is produced, then it must be assumed that the level of activity measured in the group I mutant is inadequate to produce a rate of growth sufficient to sustain viability when glycerol is the sole carbon source. If a single enzyme, inducible by either glucose or glycerol is involved, then the mutation could have occurred in the regulator gene, producing a repressor insensitive to glycerol, thus preventing growth on glycerol but allowing growth on glucose. In this case as well, the activity measured would represent residual

glucose-induced enzyme. Genetic studies and extensive purification of the enzyme produced under different conditions of growth will be required to distinguish between these possibilities.

The resistance of group II mutants to infection by phages capable of attacking the wild-type, PA-1, and mutants of group I is of considerable interest. Preliminary data (R. R. Green, personal communication) indicate that the phage are not adsorbed by group II mutants. Since adsorption depends upon the presence of specific sites in the cell wall, these mutants may be assumed to have an altered cell wall. This alteration does not affect viability since cells grow quite well in media in which phage adsorption does not occur. It is possible that the alteration in the cell wall is the result of a second mutation distinct from that involved in ability to grow on glycerol. However, the occurrence of a number of apparently identical, independent, double mutations seems less likely than the occurrence of a single mutation affecting both glycerol utilization and cell wall composition or structure. The most obvious explanation for such a single mutation is a requirement for glycerol or a product formed from it in cell wall synthesis. A glycerol-containing teichoic acid would present one possibility but these have not been reported in P. aeruginosa to our knowledge. Triglycerides or other glycerol-containing lipids may also be components of the cell wall and these compounds could be involved in phage adsorption. Based on present knowledge of the cell wall of P. aeruginosa, it appeared quite possible that rhamnose might be the compound involved in the cell wall alteration. Both Collins (30) and Eagon and Carson (26) have reported that rhamnose is a component of the cell wall of P. aeruginosa, although neither has specified its

exact location. Since rhamnose is a component of the lipopolysaccharide layer in Salmonella typhimurium (31) and a rhamnose-containing lipid is produced in large quantities by P. aeruginosa growing on glycerol or fructose (25), it is possible that rhamnose occurs in a lipopolysaccharide layer in the wall of P. aeruginosa. This layer, in E. coli, contains sites for adsorption of phages T3, T4 and T7 (32). Hauser and Karnovsky (25) showed by labelling studies that glycerol is the precursor for rhamnose synthesis in P. aeruginosa, and that fructose is an intermediate in the pathway. Neither fructose nor rhamnose was capable of replacing the yeast extract requirement of group II mutants for growth on glucose, nor did they promote growth on glycerol. This does not preclude the possibility that rhamnose is the compound involved in phage sensitivity, since the cell wall alteration may have no effect on growth; i.e., the two phenomena may have a common origin in glycerol metabolism but may result from effects on different pathways of utilization of glycerol for synthesis of cell components. The fact that these mutants lack α-glycerophosphate dehydrogenase, but have glycerol kinase activity, would tend to indicate that rhamnose, rather than glycerol-lipids, is involved in the cell wall alteration since glycerol phosphate, which can be produced by these cells is the usual precursor for glyceride synthesis. Since these mutants do not grow on a combination of glucose and glycerol, it does not appear likely that a defect in synthesis of glycerol-lipids is the primary lesion in these cells.

CHAPTER V

SUMMARY AND CONCLUSIONS

The growth of Pseudomonas aeruginosa on various substrates was characterized. The enzymes of inducible degradative pathways were contrasted with the constitutive enzymes of glucose dissimilation; evidence was cited indicating the rapid degradation of inducible enzymes on incubation in the absence of substrate, whereas the constitutive glucose enzymes did not appear to lose activity on similar treatment. The presence of glucose in the growth medium was shown to have no effect on the synthesis or activity of the inducible enzymes of the histidine degradative pathway. The enzymes of glycerol degradation, however, appeared to be subject to both repression and inhibition by glucose. The first intermediate in the pathway for histidine, urocanic acid, was found to be the inducer of histidase, the enzyme required for its own formation, as well as enzymes required for its further degradation.

Data were accumulated elucidating the pathway of glycerol catabolism for this organism. No mechanism for the active incorporation of glycerol into the cells was found to be present. α-Glycerophosphate did not support growth, presumably because of impermeability of the cells to this substrate. Analyses of cell extracts for enzymic activities showed that glycerol is first converted to L-α-glycerophosphate by glycerol kinase. The transforma-

tion of L-α-glycerophosphate to dihydroxyacetone phosphate is mediated by an NAD-independent L-α-glycerophosphate dehydrogenase. Triose phosphate isomerase effects the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate. The levels of the first two enzymes of the pathway were shown to vary markedly between induced and non-induced cells. Variation in levels of triose phosphate isomerase activity was not significant for these conditions.

Two groups of mutant organisms were isolated for the glycerol pathway. These groups were distinguished by their ability to use glucose as sole carbon source in a minimal medium. Although both groups of mutants were unable to use glycerol as substrate, only the group I mutants had retained the ability to grow in a glucose minimal medium. The mutants of the second group were able to use glucose only when yeast extract was also present in the medium. The ability to utilize mannitol was also lost in the latter group, even in the presence of yeast extract.

Enzyme data for two mutants were obtained. No appreciable loss of glycerol kinase activity was found for either mutant. α-Glycerophosphate dehydrogenase activity was observed for an extract of a group I mutant, but was absent in a similar extract of a group II mutant. Triose phosphate isomerase activity was greatly reduced for both mutants as compared to the wild type. A basal level of isomerase activity persisted in both extracts, however.

A new method for measuring glycerophosphate dehydrogenase activity in whole cells was developed for use in this study. By its use, one mutant of group I was shown to lack dehydrogenase activity.

A marked difference in susceptibility to phage was observed for

the two sets of mutants. Whereas the group I cells were readily lysed by E-1°PA-1, none of 13 strains of phage tested was able to lyse the other mutants. Preliminary data were obtained indicating that a change in the cell wall structure may be involved in the phenomenon, since phage adsorption is probably affected in group II cells.

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