GLUCOSE METABOLISM AND CATABOLITE REPRESSION IN *PSEUDOMONAS AERUGINOSA*

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GLUCOSE METABOLISM AND CATABOLITE REPRESSION IN PSEUDOMONAS AERUGINOSA

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CHAPTER I

INTRODUCTION

The aerobic pseudomonads have been of considerable biochemical interest since den Dooren de Jong, in 1926, demonstrated their ability to grow on a wide range of organic compounds as sole sources of carbon and energy (Stephenson, 1949). The metabolic versatility of this group has been thoroughly documented by Stanier, et al. (1966). Pseudomonas aeruginosa, P. fluorescens and P. putida comprise the fluorescent group. Representative strains of this group typically grew on about 80 of 146 compounds tested as sole carbon and energy source. Compounds supporting growth included paraffin hydrocarbons, alcohols, amines, polyols, mono-, di- and tricarboxylic acids, hydroxy-, amino- and keto-acids, amides, monosaccharides, hexonic acids and substituted aromatic compounds. Such wide catabolic capabilities are clearly advantageous to these ubiquitous inhabitants of soil and water.

A corollary of metabolic versatility is the existence of efficient metabolic control mechanisms governing the formation and activity of the specific enzymes required to convert each compound to central metabolites. Davis (1961) has emphasized the significance of even minute selective advantages in terms of bacterial proliferation. Indiscriminate synthesis of unnecessary enzymes would divert monomers and energy from formation of new cells. Thus, the enzyme systems responsible for such broad catabolic capabilities would be expected to

be subject to induction by their specific substrates and closely related compounds. Furthermore, the ability to degrade most of the common amino acids would not be of survival value if these capabilities were expressed under conditions requiring de novo biosynthesis of these monomers for growth. Therefore, catabolite repression might be expected to inhibit formation of these enzyme systems in spite of the presence of the appropriate inducers. This consideration also applies to substrates which are not direct precursors of macromolecules. As stated by Paigen and Williams (1970), in nature "survival may depend on utilization of the optimum substrate, for the cell that saves the best carbon source for last may well find that its neighbour has not been so forbearing" (p. 252).

Much of the current interest in the fluorescent pseudomonads as systems for biochemical genetics and metabolic control studies stemmed from the initial observations of Holloway, et al. (1963) that P. aeruginosa apparently lacked the close linkage of functionally related genes typical of Escherichia coli and Salmonella typhimurium. In the operon model of Jacob and Monod (1961), clustering of functionally related genes was intimately related to control of gene expression. Demerec (1964) emphasized the significance of Holloway's observations and stressed the importance of further comparative studies of linkage and genetic control. Lack of clustering is also typical of the eucaryote Neurospora crassa which has been the subject of extensive biochemical genetic analyses. Thus, the pseudomonads might possess genetic organization and control mechanisms more closely related to those of higher forms, including man.

The operon model of Jacob and Monod (1961) has served as the basis for understanding the control of gene expression. This model evolved from extensive biochemical and genetic investigations of lactose metabolism in E. coli. Numerous review articles have dealt with the evidence supporting the model, its generality and the significance of observed inconsistencies in other less thoroughly studied systems (e.g., Vogel and Vogel, 1967; Epstein and Beckwith, 1968; Martin, 1969). Recently a monograph devoted entirely to various aspects of the lactose (lac) operon has appeared (Beckwith and Zipser, 1970). Only the more salient features of the model will be considered in the following discussion of the lac operon.

As originally conceived, the lac operon consists of an operator (o) locus and three contiguous structural genes coding for β-galactosidase (z), galactoside permease (y) and thiogalactoside transacetylase (a), as shown in Figure 1. Galactoside permease, which mediates the entry and accumulation of β-galactosides within the cell, and β-galactosidase, which hydrolyzes lactose and other β-galactosides, are essential for lactose catabolism. Thiogalactoside transacetylase is not essential for lactose catabolism and its function is unknown. The regulator (i) gene codes for a macromolecular repressor substance which controls the expression of the structural genes (z, y and a) by interacting with the operator locus. The regulator is not part of the operon and need not be closely linked to the genes it controls since it exerts its effects through its product, the repressor. A later refinement of the model was the identification of the promoter (p) region which has been suggested as the binding site for RNA (ribonucleic acid) polymerase (Ippen et al., 1968). This element is contiguous with the

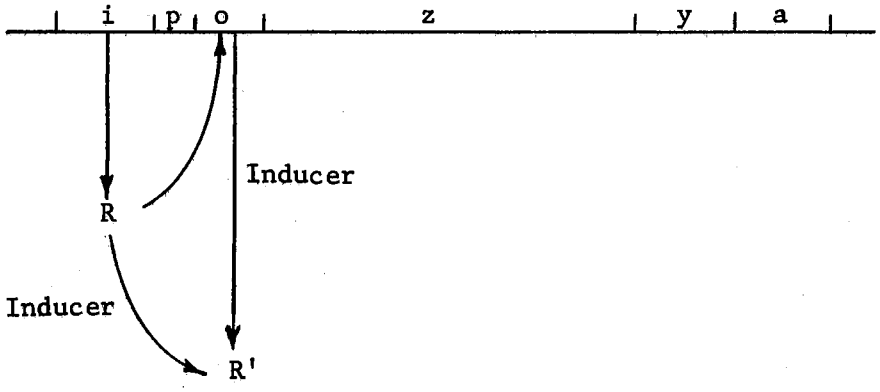


Figure 1. The Lactose Operon of Escherichia coli

Symbols:
- i - structural gene for repressor
- p - promoter
- o - operator
- z - structural gene for β-galactosidase
- y - structural gene for galactoside permease
- a - structural gene for thiogalactoside transacetylase
- R - repressor (active)
- R'- repressor-inducer complex (inactive)

operator locus and is also considered as part of the lac operon.

In the absence of an appropriate inducer, the repressor interacts with the operator and blocks transcription of the structural genes into a polycistronic messenger RNA (mRNA) thus preventing expression of the z, y and a loci. Inducers interact with the repressor and decrease its affinity for the operator. This allows transcription to proceed and the resulting polycistronic mRNA is subsequently translated into the protein products of the lac operon. Lactose is not the true inducer but depends on β-galactosidase action to form a transgalactosylation product which is the true inducer (Burstein et al., 1965). The z, y and a gene products are not synthesized in a strictly coordinate manner; that is, they are not produced in equimolar amounts, β-galactosidase being favored over thiogalactoside transacetylase. Here the lac operon is exceptional since three other operons have been shown to produce equimolar amounts of operator proximal and distal enzymes (Epstein and Beckwith, 1968).

Several types of genetic evidence support the operon model. Extensive clustering of functionally related genes is common in E. coli and S. typhimurium (Taylor, 1970; Sanderson, 1970). This has been taken as an indication of the generality of this type of control in the enterics (Demerec, 1964). More important, numerous control mutants with the predicted pleiotropic effects and dominance relationships have been characterized. Regulator (i) gene mutants of the lac operon are of two types: i^- mutants, which produce the z, y and a gene products constitutively, apparently produce a repressor which lacks affinity for the operator; i^s mutants (super-repressed), which are uninducible, apparently produce a repressor which lacks affinity for inducer.

Dominance studies in F'-lac merodiploids show that i^s is trans-dominant over i^+ and i^- and that i^+ is trans-dominant over i^- as expected of a gene which effects control via its diffusible product, the repressor. Operator constitutive (o^c) mutations, which have been attributed to loss of the repressor recognition site, are cis-dominant as predicted. Promoter mutants show pleiotropic reduction of the z, y and a gene products and are cis-dominant as expected. Such mutants are inducible, although to only low levels, indicating that i and o functions are normal. The existence of polar mutants is a further attribute of operon organization and function. Certain point mutations occurring in a structural gene can pleiotropically reduce the expression of more distal genes. The extent of reduction depends on the location of the point mutation within the gene, those further from the distal end showing greater reduction. Such mutants are apparently the result of generation of a nonsense triplet by base substitution or frameshift mutation, and the polar effects are a consequence of translation of polycistronic mRNA.

Additional support for the operon model comes from kinetic studies of mRNA and enzyme formation. The use of inhibitors of known effect has facilitated such analyses.

The strongest support has come from the isolation and in vitro characterization of the lac repressor (Gilbert and Müller-Hill, 1970). The repressor is a tetrameric protein and specifically binds to lac operator DNA (deoxyribonucleic acid). Inducers destabilize this complex and thus prevent the effective interaction of the repressor with the operator.

It should be noted that the operon model describes regulation of gene expression as a negative control mechanism. That is, expression of the z, y and a genes is the normal state of the operon, and control results from preventing this expression. The model accounts for induction and coordinate control of clustered genes in terms of regulatory genes which exert their effects on transcription to form polycistronic mRNA.

The model is readily adapted to account for end product repression of the sequential enzymes of a biosynthetic pathway. In this case, the repressor is presumably made in an inactive form. Interaction of the repressor with the end product increases its affinity for the operator and thus represses synthesis of the enzymes of the operon which function in end product formation.

Although clustering of functionally related genes is an integral part of the operon model as originally conceived, it is not essential for negative control exerted by repressors as described above. Arginine (arg) biosynthesis in E. coli depends on eight enzymes whose corresponding structural genes are located in five different regions of the chromosome. The expression of each gene is controlled by a regulator gene, arg R, which determines a repressor. This repressor has recently been purified and is a protein (Udaka, 1970). This system requires the additional assumption that each structural gene or small cluster of structural genes possesses its own operator (and probably its own promoter). Such a system composed of several operons, each controlled by the same repressor, is termed a regulon.

Negative control of gene expression is not universal in E. coli. Compelling genetic evidence has been presented for a positive control

mechanism in the L-arabinose (ara) operon (Englesberg et al., 1969). The regulator gene, ara C, produces an activator which is required for full expression of the operon's structural genes. The activator exerts its effect at an initiator locus, ara I. Two classes of regulator gene mutants are known: C^- which are uninducible (phenotypically similar to i^s of the lac operon) and C^c which are constitutive (phenotypically similar to i^-). However, C^- is recessive to C^+ and C^c in the trans position, whereas i^s is trans-dominant to i^+ and i^-. Thus, C specifies a diffusible activator. An exhaustive search for an hypothetical ara R mutant among arabinose positive revertants of an ara C^- (deletion) strain revealed only ara I constitutive mutants. These are cis-dominant low level constitutive mutations and are subject to activation by a trans C^+ allele. Various alternatives to positive control have been eliminated. Thus, it appears quite likely that the arabinose operon is under positive control.

In his now classic review of catabolite repression, Magasanik (1961) focused attention on the glucose effect in terms of its physiological significance and apparent similarity to end product repression in biosynthetic pathways. The enzymes known to be subject to glucose repression were characteristically involved in supplying the same central intermediates as glucose. In inducible systems subject to glucose repression, the effect was not entirely due to inducer exclusion by glucose. Furthermore, other substrates caused the effect suggesting that a common intermediate rather than glucose itself was responsible, hence the term catabolite repression. In general, substrates supporting higher growth rates were more repressive than those supporting lower growth rates. Substrates supporting rapid growth

presumably gave rise to high intracellular concentrations of central metabolites. Simultaneous consumption of inferior substrates would not benefit the cell and formation of the requisite enzymes would divert carbon and energy from production of new cells. Thus, one or more of the accumulated intermediates was hypothesized to effect the repression of less efficient enzyme sequences leading to the same intermediates in spite of the presence of appropriate inducers. This interpretation was supported by the observation that any condition which reduced the consumption of central intermediates without reducing their formation led to severe catabolite repression. Such conditions include starvation for an auxotrophic requirement, nitrogen or phosphate limitation in chemostat cultures, treatment with certain inhibitors and physical damage such as ^{32}P decay and ultraviolet irradiation. The possible significance of the operon model for catabolite repression was stressed.

Since 1961, considerable work has been done in various systems to elucidate the mechanism of catabolite repression and its possible relation to operon control. Recently Paigen and Williams (1970) have comprehensively reviewed the current status of catabolite repression and Magasanik (1970) has specifically reviewed catabolite repression of the lac operon.

At least four phenomena can be involved in preferential utilization of alternate substrates. These include catabolite inhibition, inducer exclusion, transient repression and catabolite repression.

Gaudy et al. (1963) first recognized the phenomenon of catabolite inhibition. This involves inhibition of existing enzymes by a more readily utilized substrate or its catabolites and is analogous to

feedback inhibition in biosynthetic pathways. The generality of this mechanism is apparent since it can be observed in natural heterogeneous populations (sewage sludge). Tsay (1968) observed rapid glucose inhibition of substrate utilization in E. coli and Achromobacter sp. growing on various substrates. Evidence was presented that the inhibition did not occur by competition for a common transport element. Similar results were obtained in E. coli by McGinnis and Paigen (1969). For lactose and galactose, inhibition was shown to occur prior to formation of the first intermediate. Thus, in cases where the first intermediate is the true inducer, catabolite inhibition may also prevent induction as a secondary effect.

The importance of inducer exclusion as a mechanism for preferential substrate utilization depends on the system under consideration and the experimental conditions. The effect of inducer exclusion is maximal when inducer permeation is induction dependent and nonsaturating concentrations of inducer are used. In E. coli, induced and constitutive expression of the lac operon are equally sensitive to glucose when induction is effected with saturating concentrations of the potent gratuitous inducer IPTG (isopropylthiogalactoside). In contrast, induced expression of the galactose (gal) operon is much more sensitive to glucose than is constitutive expression. This additional sensitivity is due to inhibition of inducer entry by glucose (Adhya and Echols, 1966).

Glucose and other compounds can also exert two effects on enzyme synthesis which are not due to inducer exclusion. Catabolite repression refers to the permanent and often weak repression observed when cells are grown in the presence of repressing substrates. For

example, in E. coli the differential rate of β-galactosidase synthesis in fully induced cells growing on glucose is typically one half that of fully induced cells growing on glycerol which is only weakly repressive. Repression by gluconate is comparable to that exerted by glucose, whereas a mixture of glucose and gluconate is more repressive. Under similar conditions, glucose 6-phosphate represses to one tenth the differential rate observed in glycerol medium. Likewise, the differential rate of constitutive β-galactosidase synthesis in glucose medium is repressed to about one half that observed in glycerol medium.

Transient repression was recognized by Moses and Prevost (1966) and Paigen (1966). Addition of glucose to most strains of E. coli grown on glycerol and exposed to a saturating concentration of inducer caused a severe transient repression of β-galactosidase synthesis. A few strains exhibit this effect when glucose is substituted for glycerol in the presence of a saturating concentration of inducer. Transient repression usually lasts for one tenth to one half of a generation, depending on the strain, and is followed by an acceleration of β-galactosidase synthesis to the differential rate characteristic of catabolite repression by glucose. The rare strains showing transient repression when glucose is substituted for glycerol are apparently mutants especially well adapted to rapid utilization of glucose (Tyler et al., 1967).

Transient repression has been observed for other enzymes and in other organisms. It appears that transient repression affects the same group of enzymes as does catabolite repression; that is, those involved in catabolism of alternate substrates.

Tyler and Magasanik (1970) have investigated the necessary and sufficient conditions for transient repression in E. coli. The effect was not unique to glucose and was observed in cells grown on substrates other than glycerol. Rapid entry of the added compound appeared to be essential although its further extensive metabolism was not required. A mutant lacking phosphohexose isomerase and glucose 6-phosphate dehydrogenase showed transient repression but not catabolite repression upon addition of glucose. Similar results were obtained with a mutant (cat^-) selected for resistance to catabolite repression exerted by glucose. This strain has apparently suffered an unidentified alteration in glucose metabolism (Rickenberg et al., 1968; Tyler et al., 1969). Thus, catabolite repression does depend on further metabolism of glucose. Evidence was also presented that transient repression required a functional enzyme I (EI) of the phosphoenolpyruvate phosphotransferase system regardless of whether or not the added compound used this system for transport. However, the relationship is not clear since other EI deficient mutants show increased sensitivity to transient repression (Pastan and Perlman, 1969).

Both transient repression and catabolite repression appear to act at the level of transcription, specifically initiation of transcription. This was deduced for the lac operon by kinetic studies of β-galactosidase synthesis using inducer removal and inhibitors of known effect (Nakada and Magasanik, 1962; Kepes, 1963; Nakada and Magasanik, 1964; Kaempfer and Magasanik, 1967; Tyler and Magasanik, 1969; Jacquet and Kepes, 1969). Recently, Varmus et al. (1970) have confirmed that transient repression prevents lac mRNA synthesis by using DNA-RNA hybridization analysis to determine lac mRNA levels directly.

Perlman and Pastan (1968) have discovered that cyclic adenosine 3', 5'-monophosphate (cAMP) relieves transient repression of β-galactosidase. Catabolite repression was also relieved by a higher cAMP concentration (Ullmann and Monod, 1968; Perlman et al., 1969). Cyclic AMP apparently stimulates initiation of lac operon transcription (Jacquet and Kepes, 1969). Direct determination of lac mRNA levels confirmed that cAMP acts at the transcription level (Varmus, et al., 1970).

In a brief review of the rapid developments regarding cAMP, Pastan and Perlman (1970) noted that as far as is known, cAMP specifically stimulates synthesis of proteins which are sensitive to transient repression and catabolite repression. Cyclic AMP effects have also been observed in other enteric organisms. It should be mentioned that cAMP appears to act at the level of translation to relieve catabolite repression of tryptophanase synthesis in E. coli (Pastan and Perlman, 1969).

Neither the regulator gene nor the operator locus (as defined genetically by interaction with the lac repressor) is required for transient repression or catabolite repression of the lac operon (Magasanik, 1970). In strains where the lac operon has been transposed and then fused by deletion with the tryptophan operon, various lac control elements can be deleted. Those strains retaining the lac promoter were sensitive to catabolite repression whereas those in which p had been deleted were insensitive to transient repression and catabolite repression (Silverstone et al., 1969; Magasanik, 1970). Point mutations in p do not usually alter sensitivity to transient repression and catabolite repression. However, second site revertants

of promoter point mutants are often insensitive to transient repression and catabolite repression. One such revertant has been shown to be extremely closely linked to the original point mutation and is presumably a further alteration of the promoter (Magasanik, 1970). The mutation to catabolite insensitivity and restored promoter function is cis-dominant.

Thus, transient repression, catabolite repression and cAMP apparently control expression of the lac operon by governing the frequency of initiation of transcription via the promoter.

A model for catabolite repression based on studies of mutants of E. coli in which the lac operon was insensitive to repression by glucose was proposed by Loomis and Magasanik (1965 and 1967). A regulator gene, cat (formerly designated CR), was thought to produce an inactive repressor which, under conditions of catabolite repression, was activated by a central metabolite, the effector. The activated repressor presumably interacted with an operator-like controlling site to prevent transcription of mRNA. However, Rickenberg et al. (1968) presented evidence that the cat$^-$ lesion was not specific to the lac operon and that it did not relieve catabolite repression exerted by glucose 6-phosphate or a mixture of glucose and gluconate. It was suggested that the cat$^-$ lesion altered glucose metabolism and prevented accumulation of the effector. Further investigation of the cat$^-$ lesion by Tyler et al. (1969) has led to the same conclusion.

The concept of an inactive repressor has been abandoned recently in favor of a model interrelating catabolite repression, promoter function and cAMP effects (Magasanik, 1970). The template specificity of RNA polymerase depends on a subunit of the enzyme known as the sigma

factor (Burgess et al., 1969). Sigma apparently facilitates the initiation of transcription at the promoter (Travers and Burgess, 1969). Catabolite-sensitive operons are hypothesized to have a unique type of promoter which requires a unique sigma factor for initiation of their transcription. Cyclic AMP is presumably required for polymerase-sigma interaction or for interaction of the polymerase-sigma complex with the promoter. Catabolite repression results from accumulation of an effector which decreases the intracellular concentration of cAMP, thus preventing initiation of transcription. As noted by Magasanik (1970), the model may be an oversimplification but is consistent with the known facts.

Regardless of the model, the existence of one or more effector metabolites must be hypothesized. However, the hypothetical effector has not been identified in any system (Paigen and Williams, 1970). Furthermore, it is unknown whether or not a given catabolite-sensitive system is subject to regulation by a single effector or how wide a range of catabolite-sensitive systems a single effector may control. Identification of the effector is complicated by interdependent metabolic conversions and lack of knowledge regarding their associated control mechanisms. In addition, strain differences may confound extrapolation and comparative analyses.

The lack of extensive clustering of functionally related genes coding for biosynthetic enzymes in Pseudomonas has been confirmed in more detailed analyses of individual pathways (Holloway, 1969). However, linkage of related genes is not totally lacking since isolated clusters containing two or three genes have been demonstrated for several pathways. In the few cases where parallel control studies have

been done, it is apparent that clustering does have significance in terms of control of gene expression.

Marinus and Loutit (1969a) have demonstrated that the structural genes for two enzymes of isoleucine-valine biosynthesis (acetohydroxy acid synthetase and reductoisomerase) are contiguous in P. aeruginosa. A possible operator mutation with pleiotropic effects mapped outside but close to the cluster. The two enzymes were subject to coordinate multivalent repression (Marinus and Loutit, 1969b).

The enzymes of tryptophan biosynthesis in P. putida are coded by six genes which occur as two unlinked clusters of three and two genes each and a single gene unlinked to either cluster (Chakrabarty, et al., 1968; Gunsalus et al., 1968). The three-gene cluster specifies anthranilate synthetase, phosphoribosyl transferase and indoleglycerolphosphate synthetase which are noncoordinately repressed by tryptophan (Crawford and Gunsalus, 1966). Tryptophan synthetase A and B proteins are coded by the two-gene cluster and are induced by indoleglycerolphosphate, the substrate of tryptophan synthetase. A possible operator mutation which led to constitutive synthesis of the A and B proteins was closely linked to the two-gene cluster. The isolated gene codes for phosphoribosyl anthranilate isomerase.

The convergent pathways by which aromatic compounds are degraded in Pseudomonas have been analyzed extensively. In the β-ketoadipate pathway, various aromatic compounds are degraded by specific peripheral sequences to either protocatechuate or catechol. Two parallel central sequences convert these to β-ketoadipate enol-lactone which is further degraded to succinate and acetyl coenzyme A. Ornston (1966) has elucidated the control of the enzymes of the central sequences in P. putida.

Four units of control were defined by coordinate enzyme synthesis and/or induction specificity. The enzymes were also subject to catabolite repression by succinate or glucose. The multi-enzyme control units consisted of carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase and β-ketoadipate enol-lactone hydrolase, which were induced by β-ketoadipate, and muconate lactonizing enzyme and muconolactone isomerase, which were induced by muconate. It is a unique consequence of product induction by β-ketoadipate that the two enzymes converting carboxymuconate to β-ketoadipate enol-lactone are synthesized gratuitously during growth on substrates degraded via catechol. The β-ketoadipate pathway also provides examples of sequential induction.

Based on Ornston's work, Kemp and Hegeman (1968) demonstrated identical control patterns in P. aeruginosa. Transductional analysis confirmed the prediction that genes specifying coordinately controlled enzymes would be closely linked. An additional loose clustering of related genes was noted.

Wheelis and Stanier (1970) have recently demonstrated the expected close linkage of the coordinately expressed genes of this system in P. putida. The general loose clustering of related but noncoordinately expressed genes was also noted. It was suggested that this "supraoperonic" clustering might be a selective advantage in transferring functionally related genes on small fragments of DNA, which is characteristic of bacterial sexual mechanisms. An interesting example of this, which also demonstrated close linkage of the coordinately expressed mandelate genes in P. putida, was the transduction of the entire group of genes into another strain in which these genes are

apparently absent (Chakrabarty et al., 1968).

Thus, in a recent review of Pseudomonas genetics, Holloway (1969) concluded that, although further control studies are limited by the lack of merodiploid strains, the control mechanisms observed are consistent with operon-like control. However, the patterns of control imposed on various pathways are quite different in Pseudomonas compared to the enterics. Indeed, Canovas et al. (1967) have suggested that control mechanisms have probably evolved independently and therefore represent useful taxonomic characteristics. This was supported by the demonstration of radically different control patterns for the β-ketoadipate pathway in taxonomically distinct organisms.

The present study was undertaken in an attempt to extend the analysis of control of gene expression in P. aeruginosa. As noted previously, the effector(s) of catabolite repression is not known for any system. Since glucose generally serves as an efficient source of the effector, it seemed reasonable that mutants unable to degrade glucose might show altered catabolite repression and thus provide information regarding the identity of the effector metabolite. Such mutants would also be useful for studying the control of glucose metabolism and the linkage of the corresponding structural genes in P. aeruginosa.

Histidase, the first enzyme in histidine catabolism, has been useful in kinetic studies of induced enzyme synthesis since a simple, sensitive whole cell assay was possible (Hartwell and Magasanik, 1963; Schlesinger and Magasanik, 1965). Lessie and Neidhardt (1967b) have examined the control of histidine catabolism in P. aeruginosa. Histidase, urocanase and the formiminoglutamate degrading enzyme, which catalyze the first, second and fourth steps of the pathway,

respectively, were inducible. Convincing evidence was presented that urocanate, the first intermediate, was the true inducer for these enzymes. This was recently proved using a mutant lacking histidase (Newell and Lessie, 1970). Urocanate also appears to be the true inducer in Aerobacter aerogenes, S. typhimurium and P. putida (Schlesinger et al., 1965; Brill and Magasanik, 1969; Wheelis and Stanier, 1970). However, histidine is apparently the true inducer in Bacillus subtilis (Chasin and Magasanik, 1968).

Lessie and Neidhardt (1967b) also noted that the three enzymes of histidine catabolism which were investigated in P. aeruginosa were subject to catabolite repression by a variety of substrates including succinate and glucose. Histidase and urocanase were reported to be coordinately repressed whereas repression of the formiminoglutamate degrading enzyme was noncoordinate with these. Thus, histidase was chosen as an inducible catabolic enzyme for monitoring the effect of different glucose lesions on catabolite repression of histidine degradation in the present study.

The pathways by which glucose and related compounds are metabolized in Pseudomonas are summarized in Figure 2. These organisms degrade glucose primarily by the Entner-Doudoroff (ED) pathway and to a lesser extent by the hexose monophosphate (HMP) pathway (Wang et al., 1959). Several enzymes of the Embden-Meyerhof-Parnas (EMP) pathway can be demonstrated in cell-free extracts but a catabolically functional EMP pathway is lacking. Wood and Schwerdt (1954) were unable to detect phosphofructokinase, an essential EMP pathway enzyme, in extracts of P. putida (formerly fluorescens) although fructose diphosphate aldolase was present. P. aeruginosa ATCC 7700 was reported to lack fructose

Figure 2. Reactions Involved in Metabolism of Glucose and Glycerol.

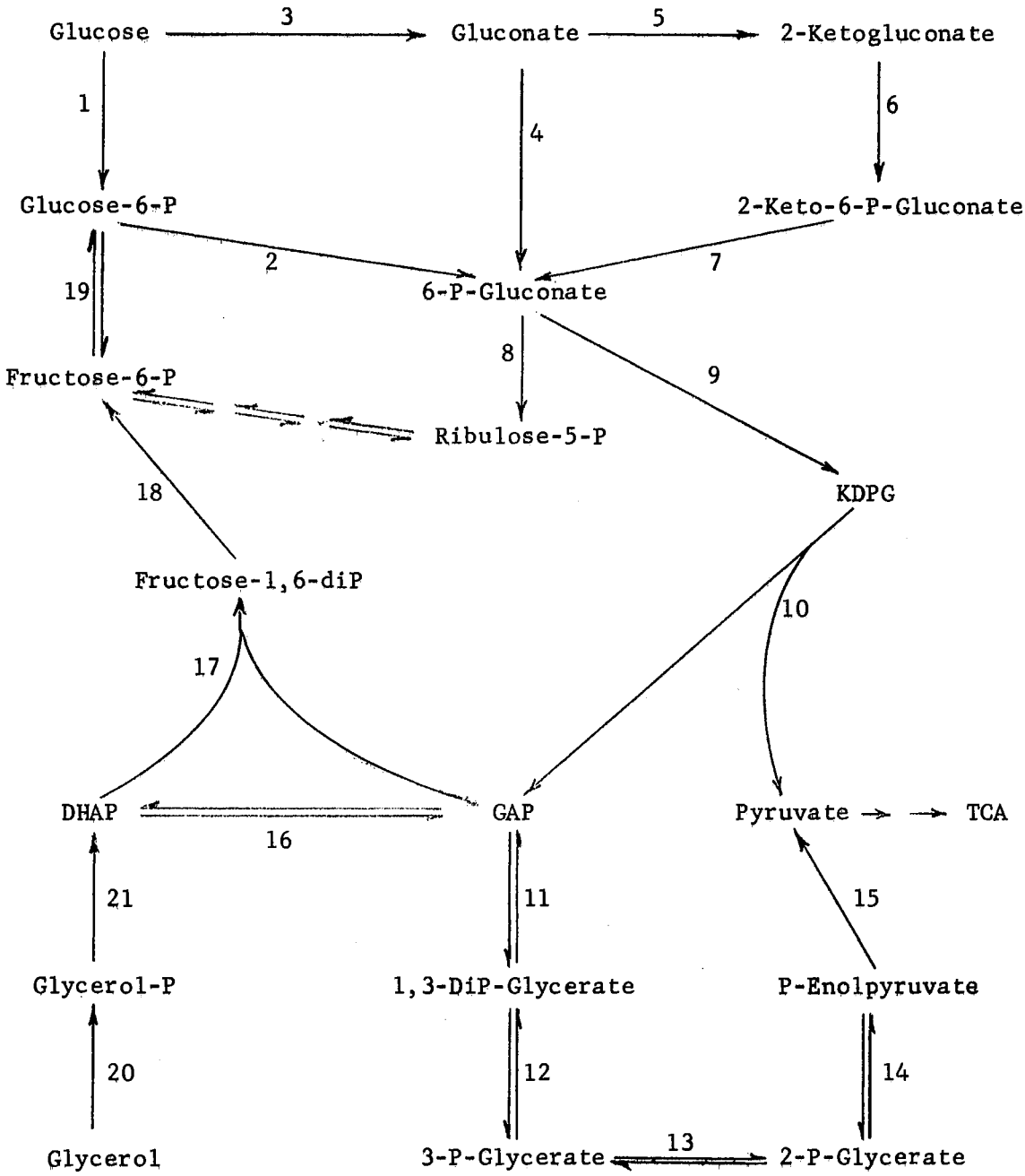
Enzymes and abbreviations are listed below. Enzyme numbers correspond to numbered reactions shown in figure. Directions shown for reactions are those found to be important for growth of Pseudomonas aeruginosa, PA-1, in the present study.

Abbreviations:

KDPG, 2-keto-3-deoxy-6-phosphogluconate
DHAP, dihydroxyacetone phosphate
GAP, glyceraldehyde 3-phosphate

Enzymes:

1. Glucokinase
2. Glucose 6-phosphate dehydrogenase
3. Glucose dehydrogenase
4. Gluconokinase
5. Gluconate dehydrogenase
6. 2-ketogluconokinase
7. 2-keto-6-phosphogluconate reductase
8. 6-phosphogluconate dehydrogenase
9. 6-phosphogluconate dehydrase
10. KDPG aldolase
11. Glyceraldehyde 3-phosphate dehydrogenase
12. Phosphoglycerate kinase
13. Phosphoglycerate mutase
14. Enolase
15. Pyruvate kinase
16. Triose phosphate isomerase
17. Fructose diphosphate aldolase
18. Fructose diphosphatase
19. Phosphohexose isomerase
20. Glycerol kinase
21. L-α-glycerophosphate dehydrogenase


Glucose
3
Gluconate
5
2-Ketogluconate
1
4
6
Glucose-6-P
2-Keto-6-P-Gluconate
2
7
19
6-P-Gluconate
8
9
Fructose-6-P
Ribulose-5-P
18
KDPG
Fructose-1,6-diP
10
17
DHAP
16
GAP
Pyruvate
TCA
21
11
15
Glycerol-P
1,3-DiP-Glycerate
P-Enolpyruvate
20
12
14
13
Glycerol
3-P-Glycerate
2-P-Glycerate

diphosphate aldolase, the other essential EMP pathway enzyme (Lessie and Neidhardt, 1967a). However, Tiwari and Campbell (1969) demonstrated that the only EMP pathway enzyme missing in P. aeruginosa ATCC 9027 was phosphofructokinase.

The route of glucose degradation is complicated by the presence of glucose dehydrogenase and gluconate dehydrogenase which catalyze direct glucose oxidation to gluconate and 2-ketogluconate (Stokes and Campbell, 1951). This potentially allows glucose to be degraded by three pathways which diverge from glucose and converge at 6-phosphogluconate. In P. putida, gluconate and 2-ketogluconate are phosphorylated by specific kinases yielding 6-phosphogluconate and 2-keto-6-phosphogluconate, respectively (Narrod and Wood, 1956). The latter intermediate is subsequently reduced to 6-phosphogluconate (Frampton and Wood, 1961). Analogous enzymes presumably exist in P. aeruginosa. Conversion of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate (KDPG) and direct cleavage of the latter to pyruvate and glyceraldehyde 3-phosphate (GAP) are mediated by the two key ED pathway enzymes, 6-phosphogluconate dehydrase and KDPG aldolase. GAP is further degraded to pyruvate via the triose phosphate (TP) pathway (lower EMP sequence). 6-Phosphogluconate is also metabolized via the HMP pathway to yield biosynthetic intermediates and allow hexose phosphate resynthesis.

Hamilton and Dawes (1960) reported that extracts of P. aeruginosa grown on organic acids contained only basal levels of the glucose catabolic enzymes and that they were induced by exposure of the cells to glucose. This was confirmed by von Tigerstrom and Campbell (1966) who showed that the levels of glucokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase

and KDPG aldolase were very low or undetectable in extracts of P. aeruginosa grown on α-ketoglutarate or acetate; high levels of these activities were present in extracts of glucose-grown cells. Glucose dehydrogenase was only slightly increased from its relatively low basal level during growth on glucose. Similar results were obtained by Lessie and Neidhardt (1967a) who also noted the induction of the main pathway enzymes by gluconate and glycerol. The latter observation is surprising, especially in view of the apparent absence of fructose diphosphate aldolase in their strain.

Tiwari and Campbell (1969) extended these observations by showing that the ability to convert pentose phosphate to hexose phosphate, which depends on several enzymes of the HMP pathway, was also induced to higher levels in glucose-grown cells. Furthermore, the levels of the TP pathway enzymes were similar in extracts of succinate-grown and glucose-grown cells, indicating their apparent constitutive nature.

Preliminary to characterization of the enzyme lesions of glucose mutants isolated in the present study, analyses of the EMP pathway lesion and control of glucose catabolic enzyme synthesis in the wild type were necessary. This included an investigation of the relation between glycerol catabolism and glucose metabolism, since glucose mutants might clarify such a relationship.

CHAPTER II

MATERIALS AND METHODS

Bacteria and Bacteriophages

P. aeruginosa, strain 1, (designated PA-1) and its transducing phage F116 were originally obtained from B. W. Holloway, Monash University, Clayton, Australia. All glucose mutants were derived from PA-1 during the present study. Phage PØ-6 was supplied by R. R. Green of this laboratory. *E. coli* B was from the stock culture collection of J. W. Drake, University of Illinois.

Media

Minimal medium was a modification of M-9 medium (Roberts et al., 1957) and contained (amounts per liter): $Na_2HPO_4 \cdot 7H_2O$, 8.2 g; KH_2PO_4, 2.7 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; NH_4Cl, 1.0 g; $FeSO_4$, 0.1 per cent solution, 0.5 ml; distilled water to volume. Histidine minimal medium was prepared as a single solution, adjusted to pH 7 with NaOH pellets, and sterilized by Millipore filtration (HA, 0.45 μm pore size). All other carbon sources were autoclaved or filter-sterilized separately as concentrated solutions and added to a final concentration of 0.5 per cent unless otherwise indicated. Nutrient broth (Difco) was rehydrated as suggested by the manufacturer.

Plates contained 20 ml of medium solidified with 2.0 per cent agar (Difco) unless otherwise indicated. Sufficient Bacto-agar (Difco) was

added to nutrient agar (Difco) to give a final concentration of 2.0 per cent. In some mutant isolation experiments, glucose minimal medium plates were supplemented with various concentrations of yeast extract (Difco)

Media for Pseudomonas phage propagation and storage have been described by Holloway et al. (1962). Phage broth contained (g per liter): nutrient broth, 8.0; yeast extract, 5.0; NaCl, 5.0; distilled water to volume. Phage plates contained 35 ml of bottom layer agar, which was phage broth solidified with 1.1 per cent agar. Phage broth solidified with 0.65 per cent agar was dispensed in 2.5 ml portions into tubes and used as top layer agar for soft agar overlays.

Lactate phage agar (Sutter et al., 1963) was used for titration of phage suspensions and contained (amounts per liter): NaCl, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $NH_4H_2PO_4$, 1.0 g; K_2HPO_4, 1.0 g; sodium lactate, 60 per cent syrup, 2.0 ml; distilled water to volume. Bottom and top layer agars were solidified with 1.1 per cent and 0.65 per cent agar, respectively. These were distributed in amounts of 35 ml per plate and 2.5 ml per tube, respectively.

Growth of Bacteria

Stock cultures were maintained on nutrient agar slants by periodic transfer; once grown, the cultures were stored at 0 C. All cultures were grown at 37 C. Liquid cultures were grown in 18 mm tubes containing 6.0 ml of medium or in flasks containing a volume of medium not more than one tenth of the nominal flask volume. Liquid cultures were aerated on a reciprocal shaker during incubation. Growth was measured as optical density at 540 nm against an appropriate blank with a

Coleman Junior Spectrophotometer, Model D; an optical density of 0.220 was equivalent to 10^9 viable cells per ml.

Minimal salts medium (i.e., without added carbon source) was used for diluting and washing bacterial suspensions. When physiologically active cells were desired, the cells were washed by centrifugation and resuspension at room temperature, otherwise the cells were harvested by centrifugation at 0 C.

Preparation and Titration of Phage Plate Stocks

Phage plate stocks were prepared by a modification of the procedure of Swanstrom and Adams (1951). One drop of a culture of the desired host strain growing exponentially in nutrient broth and 0.1 ml of phage suspension containing approximately 5×10^6 plaque-forming units (PFU) per ml were mixed in 2.5 ml of molten (47 C) top layer phage agar and poured over the bottom layer phage agar plate. After 12 to 18 hours incubation at 37 C, the phage were soaked off by flooding the plates with 5.0 ml of phage broth. After 30 minutes, the phage broth was pipetted from the plates and contaminating cells and soft agar were removed by centrifugation. Residual cells were removed from the phage suspensions by Millipore filtration (HA, 0.45 μm pore size). This procedure yielded phage plate stocks with titers of approximately 10^{10} PFU per ml for phage F116 propagated on PA-1 and its glucose-negative derivatives.

Phage plate stocks were titered by the soft agar overlay method described above. Lactate phage agar was used since the plaques were more readily visible than on phage agar. PA-1 was used as host for all phage titrations. The phage suspensions were diluted in phage broth

such that 0.1 ml of an appropriate dilution yielded 30 to 300 individual plaques.

Mutant Isolation Procedures

Mutagenesis

Ethyl Methanesulfonate (EMS)

A prolonged EMS procedure for isolation of auxotrophs at high frequency without subsequent enrichment (Nečásek et al., 1967) was modified for use with P. aeruginosa. Nutrient broth-grown cells were washed and resuspended to 10^9 cells per ml in 0.067 M phosphate buffer, pH 7.2. EMS (0.065 ml) was added to 5 ml of cell suspension in a 50 ml flask to a final concentration of 0.1 M. The cells were incubated without shaking at 22 C for 16 hours.

N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

A modification of the procedure of Adelberg et al. (1965) was used. Glucose-grown cells were washed and resuspended to 10^9 cells per ml in 0.05 M acetate buffer, pH 6.0. A 1 mg per ml solution of NTG was prepared fresh in the same buffer and 0.5 ml was added to 4.5 ml of cell suspension (final concentration 100 µg per ml). The cells were incubated on a shaker at 37 C for 30 minutes and then washed in minimal medium.

Phenotypic Lag and Nuclear Segregation

EMS-treated cells were diluted 10-fold directly into minimal medium and 0.1 ml aliquots of the dilution were subcultured into a series of tubes containing 5.9 ml of nutrient broth. In the initial

NTG mutant isolations, washed mutagenized cells were resuspended to 5 ml in minimal medium and 0.1 ml aliquots were subcultured into a series of tubes containing 5.9 ml of histidine minimal medium. In both cases the survivors were grown to stationary phase. Mutants from different growth tubes of a series were assumed to be of independent origin.

In later NTG mutant isolations, washed mutagenized cells were resuspended in nutrient broth and incubated on a shaker at 37 C for 2 to 3 hours. The cells were then washed in minimal medium.

Enrichment

D-cycloserine (DCS)

Cultures of NTG-treated cells which had been grown to stationary phase in histidine minimal medium were washed and resuspended to 10^9 cells per ml in glucose minimal medium. The cells were then incubated on a shaker at 37 C for 1 hour. DCS was made up fresh to 1 mg per ml in glucose minimal medium and equal volumes (2 ml) of cell suspension and DCS were mixed (final concentration 500 µg per ml). The cultures were returned to the shaker and incubation was continued for 6 hours. The cells were then washed and resuspended to 2 ml in minimal medium. Samples of 1 ml of cell suspension were added to 5 ml of histidine minimal medium for each culture and grown to stationary phase.

D-cycloserine-carbenicillin (DCS-CAR)

NTG-treated cells which had segregated in nutrient broth for 2 to 3 hours were washed and resuspended in glucose minimal medium to 10^9 or fewer cells per ml. The cells were diluted 5-fold into glucose minimal medium and 3 ml of cell suspension were added to 3 ml of a freshly

prepared solution containing 1 mg per ml DCS and 200 µg per ml CAR in glucose minimal medium (final concentrations 500 µg per ml DCS and 100 µg per ml CAR). The cells were incubated on a shaker at 37 C for 12 hours and then washed and resuspended to 6 ml in minimal medium.

In a later modification of this procedure, the cells were washed in distilled water after enrichment in order to lyse osmotically fragile forms which were otherwise protected by the high salts concentration of minimal medium (Lessie and Whiteley, 1969). For isolation of mutants unable to grow on gluconate, glucose was replaced by gluconate in the above procedure.

Detection of Presumptive Mutants

Replica Plating

When cultures were screened for mutants immediately after a growth step, appropriate dilutions were made directly and 0.1 ml samples were spread on either nutrient agar or lactate minimal agar master plates. Cultures not grown immediately prior to screening were titered by spreading 0.1 ml aliquots of a 10-fold dilution series on lactate minimal agar plates. The culture was kept at 4 C until the appropriate dilution was known and additional lactate minimal agar master plates were prepared.

Colonies appearing on nutrient agar master plates were replicated to glucose, glycerol, and histidine minimal agar plates by the methods of Lederberg and Lederberg (1952). Those on lactate master plates were replicated to glucose or gluconate minimal agar plates. Presumptive mutant colonies were recognized by their failure to grow on the replica plates.

Substrate-Limited Colonies

Mutants unable to grow on glucose were also detected directly on glucose minimal agar plates containing 0.025 per cent yeast extract. Appropriate dilutions were made directly or determined as described above and 0.1 ml samples were spread on glucose-yeast extract plates. Glucose catabolic mutants appeared as thin, spreading, yeast extract-limited colonies.

Confirmation of Mutant Type

A small sample of the presumptive mutant colony was picked from the master plate or glucose-yeast extract plate with an inoculating needle and transferred to points on glucose or gluconate and lactate minimal agar plates. As many as 100 presumptive mutants could be tested on a single set of plates by placing a graph paper template below the plates in order to provide an orderly array of point inoculations. Stock cultures of the confirmed mutants were prepared by transferring a portion of the colony from the lactate minimal agar test plate to a nutrient agar slant.

Transduction

A modification of the semi-quantitative spot plate transduction procedure of Murphy and Rosenblum (1964) was used. Recipient cells were washed from fresh nutrient agar slants with 2 ml of minimal medium and 0.1 ml aliquots were spread on glucose minimal medium plates. One drop of each donor phage plate stock was spotted at a marked location on the surface of each plate. Phage suspensions were also spotted on nutrient agar plates without cells to test the sterility of the phage

plate stocks. The spots were allowed to soak in before the plates were incubated at 36 C. Transductants were scored after approximately three days incubation. Areas of the plates not receiving phage served as a control for reversion of the recipient strain.

Chemicals

2-Keto-3-deoxy-6-phosphogluconate (KDPG, barium salt) was the generous gift of M. Roseman and W. A. Wood, Michigan State University. The barium salt was converted to the free acid by ion exchange with excess Dowex 50 (H^+ form). Carbenicillin (CAR) was a gift from Beecham Pharmaceuticals. D-cycloserine (DCS) was obtained from Mann Research Laboratories. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was the product of Aldrich Chemical Company. Ethyl methanesulfonate (EMS) was purchased from Eastman Kodak Company. Liquid scintillation fluors were obtained from Amersham/Searle. Glucose 6-phosphate, cyclic adenosine 3',5'-monophosphate (cAMP), 3(4,5 dimethyl thiazolyl 1-2) 2,5 diphenyl tetrazolium bromide (MTT) and phenazine methosulfate were the products of Nutritional Biochemicals Corporation.

The following chemicals were purchased from Calbiochem: N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), tris(hydroxymethyl)aminomethane (Tris), adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), nicotinamide adenine dinucleotide (NAD^+), dihydronicotinamide adenine dinucleotide (NADH), dihydronicotinamide adenine dinucleotide phosphate (NADPH), uniformly labeled ^{14}C-glucose, and the diethyl acetal barium salt of D,L-glyceraldehyde 3-phosphate (GAP) which was converted to the free acid by the manufacturer's suggested procedure.

Fructose 6-phosphate, fructose 1,6-diphosphate, 6-phosphogluconate (tri-monocyclohexylammonium salt), 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate (tricyclohexylamine salt) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were obtained from Sigma Chemical Company. The following purified enzymes which were used as reagents in many of the enzyme assays were also purchased from Sigma Chemical Company: glucose 6-phosphate dehydrogenase (baker's yeast, type XV), fructose 1,6-diphosphate aldolase (rabbit muscle), GAP dehydrogenase (rabbit muscle), L-α-glycerophosphate dehydrogenase (rabbit muscle) and lactic dehydrogenase (rabbit miscle, type II).

All other chemicals were of the highest quality commercially available.

Chemical Analyses

Glucose

Glucose was determined enzymatically using the manufacturer's suggested glucostat procedure (Worthington Biochemical Corporation). Samples containing 0.05 to 0.3 mg of glucose were made up to 1.0 ml with distilled water and 9.0 ml of glucostat reagent were added. After exactly 10 minutes, the reaction was terminated with one drop of 4N HCl. The tubes were allowed to stand at least 5 minutes and then the optical density was determined at 440 nm. A water blank and glucose standards were always run with the unknown samples.

Protein

The protein content of cell-free extracts was determined by the method of Sutherland et al. (1949). Samples containing 30 to 90 µg

of protein were made up to 1.0 ml with distilled water. Five ml of freshly prepared reagent containing 1.0 ml of 4 per cent sodium potassium tartrate and 1.0 ml of 2 per cent $CuSO_4 \cdot 5H_2O$ per 100 ml of 4 per cent Na_2CO_3 were added. After 40 minutes, 0.5 ml of a two-fold dilution of 2N phenol reagent (Fisher Scientific Company) in water was added and mixed immediately. The tubes were allowed to stand at least 15 minutes and then the optical density was determined at 660 nm. A water blank and bovine serum albumin (Sigma Chemical Company) standards containing 30, 60, and 90 µg of protein were always run with the unknown samples.

Gregory and Sajdera (1970) reported that certain buffers can interfere with protein determination by the Folin-Ciocalteau method. All extracts used in the present study were prepared in 0.02 M Tris buffer, pH 7.5. At the concentrations encountered in protein determination, Tris alone gave no color. However, Tris did slightly enhance color formation due to protein; this effect was proportional to the concentration of Tris. Therefore, the protein concentration of low protein extracts was slightly overestimated and led to slightly lower enzyme specific activities. Under the worst conditions, the error in protein concentration was only 8 per cent, which was considered insignificant and was not corrected.

Preparation of Cell-free Extracts

Cells were grown with aeration at 37 C to late exponential phase in 200 ml of the desired medium, washed with saline (0.85 per cent NaCl) at 0 C and the pellets were frozen at -20 C. In cases where mutants could not be grown on the desired substrates, the cells were

grown on 0.5 per cent lactate minimal medium to late exponential phase, washed with minimal medium at room temperature and resuspended to volume in minimal medium. The desired carbon source was added to 0.5 per cent and the incubation was continued. After four hours exposure to the substrate, the cells were washed in saline at 0 C and the pellets were frozen at -20 C.

Frozen cell pellets were thawed in 5.0 ml of 0.02 M Tris buffer, pH 7.5. The cells were broken with a Bronson sonifier by exposure to about five 15-second bursts of sonic energy with intermittent cooling in an ice bath. Small, thin-walled plastic beakers were quite useful for this procedure.

Residual whole cells were removed by centrifugation at 0 C. The opalescent supernatants were poured off into chilled tubes and kept at 0 C. These crude extracts were used directly for enzyme assays.

Enzyme Assays

All enzyme assays were performed spectrophotometrically on a Cary, Model 14 dual beam recording spectrophotometer (Applied Physics Corporation). For each assay, the blank and experimental cuvettes differed only by the substitution of water for the substrate in the former. The whole cell histidase assays and direct KDPG aldolase assays were carried out in a total volume of 1.0 ml in microcuvettes. All other assays were performed in a total volume of 3.0 ml. Reagents were added in the order of decreasing stability. The cuvette contents were mixed by inversion prior to addition of reagent enzymes or crude extract and following addition of the last component of the reaction mixture. Reactions were started by the addition of substrate except in

the cases of glucose dehydrogenase and gluconate dehydrogenase which were started by the addition of PMS (phenazine methosulfate). Most enzyme assays were linked to pyridine nucleotide reduction or oxidation. In the latter case, the position of the cuvettes was reversed so that the rate of NAD(P)H oxidation was recorded as an increase in optical density. All assays were performed at room temperature (approximately 22 C). Appropriate controls were run to insure that substrates and purified enzymes used as reagents were not contaminated with interfering substances. GAP dehydrogenase was measured immediately after extract preparation since both NAD^+- and $NADP^+$-linked activities appeared to be relatively unstable.

A unit of enzyme was defined as that amount required to convert one μ mole of substrate to product(s) per minute under the specified conditions. The following millimolar extinction coefficients at the indicated wave lengths were used to calculate enzyme specific activities: reduced pyridine nucleotides at 340 nm, 6.2 (Dawson et al., 1959); formazan product of MTT reduction at 560 nm, 9.9 (Sowerby and Ottaway, 1966); phosphoenolpyruvate at 230 nm, 3.0 (Wold and Ballou, 1957); urocanate at 277 nm, 18.8 (Hartwell and Magasanik, 1963).

Glucose Dehydrogenase

The L-α-glycerophosphate dehydrogenase assay of Lin et al. (1962) was adapted for assaying glucose dehydrogenase. The reaction mixture contained: 66.7 mM Tris buffer, pH 7.5; 10.0 mM KCN; 0.24 mM MTT; 2.5 mM glucose; 0.33 mM PMS. The reaction was dependent upon PMS.

Gluconate Dehydrogenase

Gluconate dehydrogenase was assayed by the same method as glucose dehydrogenase except that glucose was replaced by 2.5 mM gluconate in the reaction mixture.

Glucokinase

Glucokinase was assayed by the method of Tiwari and Campbell (1969). The reaction mixture contained: 66.7 mM Tris buffer, pH 8.0; 10.0 mM $MgCl_2$; 2.5 mM ATP; 0.25 mM $NADP^+$; approximately 0.3 units of glucose 6-phosphate dehydrogenase; 2.5 mM glucose.

Gluconokinase

Gluconokinase was assayed by the same method as glucokinase except that 6-phosphogluconate dehydrogenase and 2.5 mM gluconate were substituted for glucose 6-phosphate dehydrogenase and glucose. Commercial 6-phosphogluconate dehydrogenase was not suitable for this assay. However, Fraenkel and Levisohn (1967) noted that extracts of *E. coli* grown on a glucose-supplemented complex medium contained high levels of 6-phosphogluconate dehydrogenase and only trace levels of gluconokinase. Therefore, 6-phosphogluconate dehydrogenase was supplied as 0.1 ml of an extract of *E. coli* B grown on nutrient broth containing 0.5 per cent glucose. This corresponded to approximately 0.1 unit of the enzyme. The observed rates were corrected for the trace levels of gluconokinase present in the *E. coli* B extracts which were approximately 2 per cent of the observed 6-phosphogluconate dehydrogenase activities.

Glucose 6-Phosphate Dehydrogenase

Glucose 6-phosphate dehydrogenase was assayed by the method of Tiwari and Campbell (1969). The reaction mixture contained: 66.7 mM Tris buffer, pH 8.0; 5.0 mM $MgCl_2$; 0.25 mM $NADP^+$; 2.5 mM glucose 6-phosphate.

6-Phosphogluconate Dehydrogenase

6-Phosphogluconate dehydrogenase was assayed by the same method as glucose 6-phosphate dehydrogenase except that 2.5 mM 6-phosphogluconate was substituted for glucose 6-phosphate.

6-Phosphogluconate Dehydrase-KDPG Aldolase

The coupled 6-phosphogluconate dehydrase-KDPG aldolase assay of von Tigerstrom and Campbell (1966) was modified. The reaction mixture contained 66.7 mM Tris buffer, pH 7.5; 2.5 mM $MgCl_2$; 0.25 mM NADH; approximately 6 units of lactic dehydrogenase; 2.5 mM 6-phosphogluconate. The reaction rate was limited by 6-phosphogluconate dehydrase.

The sensitivity of the assay was doubled by inclusion of approximately 6 units of L-α-glycerophosphate dehydrogenase which allowed detection of the GAP as well as the pyruvate produced by KDPG cleavage. Extracts of glucose mutants were examined for 6-phosphogluconate dehydrase by the double sensitivity assay in the presence of excess KDPG aldolase. The KDPG aldolase was provided in the form of 0.1 ml of an extract of *E. coli* B grown on nutrient broth containing 0.5 per cent glucose. As noted by Fraenkel and Levisohn (1967), such extracts contain high levels of KDPG aldolase but no detectable 6-phosphogluconate dehydrase activity. The added *E. coli* B extract corresponded to

approximately 0.16 units of KDPG aldolase. The procedure used to test for the presence of KDPG aldolase in extracts of glucose mutants was similar except that the E. coli B extract was replaced by an extract of lactate-grown, glucose-induced 720 cells. Mutant 720 lacked KDPG aldolase but retained 6-phosphogluconate dehydrase. Thus, the 720 extract served to generate KDPG from 6-phosphogluconate in the reaction mixture. KDPG aldolase specific activities obtained by this procedure were interpreted qualitatively since this method underestimated the activity observed by direct KDPG aldolase assay.

KDPG Aldolase

KDPG aldolase was assayed by a modification of the coupled assay described above. The reaction mixture contained 50 mM Tris buffer, pH 7.5; 2.5 mM $MgCl_2$; 0.38 mM NADH; approximately 6 units of lactic dehydrogenase; 2.5 mM KDPG.

Phosphohexose Isomerase

Phosphohexose isomerase was assayed by a modification of the method of Gale and Beck (1967). The reaction mixture contained: 66.7 mM Tris buffer, pH 8.0; 5.0 mM $MgCl_2$; 0.25 mM $NADP^+$; approximately 0.3 units of glucose 6-phosphate dehydrogenase; 2.5 mM fructose 6-phosphate.

Fructose Diphosphatase

Fructose diphosphatase was assayed by the same method as phosphohexose isomerase except that 2.5 mM fructose 1,6-diphosphate was substituted for fructose 6-phosphate. The extract supplied the necessary excess phosphohexose isomerase activity.

Phosphofructokinase

The assay of Ling et al. (1966) was modified to detect production of GAP from fructose 6-phosphate, in the presence of excess fructose 1,6-diphosphate aldolase and GAP dehydrogenase, by measurement of the reduction of NAD^+. The reaction mixture contained: 66.7 mM Tris, pH 8.0; 50 mM KCl; 16.7 mM Na_2HAsO_4; 5.0 mM $MgCl_2$; 2.5 mM ATP; 0.25 mM NAD^+; approximately 4.6 units of fructose diphosphate aldolase; approximately 10 units of GAP dehydrogenase; 2.5 mM fructose 6-phosphate.

Fructose Diphosphate Aldolase

The assay of Groves et al. (1966) was modified to detect production of GAP from fructose 1,6-diphosphate in the presence of excess GAP dehydrogenase by reduction of NAD^+. The reaction mixture contained: 66.7 mM Tris, pH 7.5; 100 mM KCl; 16.7 mM Na_2HAsO_4; 10.0 mM freshly neutralized cysteine; 0.25 mM NAD^+; approximately 10 units of GAP dehydrogenase; 2.5 mM fructose 1,6-diphosphate.

GAP Dehydrogenase

Both NAD^+- and $NADP^+$-linked GAP dehydrogenase activities were assayed by the methods of Tiwari and Campbell (1969). The reaction mixtures contained: 66.7 mM Tris buffer, pH 7.5; 20.0 mM NaF; 16.7 mM Na_2HAsO_4; 10.0 mM freshly neutralized cysteine; 0.25 mM NAD^+ or $NADP^+$; 1.67 mM D,L-GAP.

Phosphoglycerate Kinase

Phosphoglycerate kinase was assayed by a modification of the procedure of Campbell et al. (1966). The reaction mixture contained: 66.7 mM Tris buffer, pH 7.5; 2.5 mM $MgCl_2$; 2.5 mM ATP; 0.25 mM NADH; approximately 10 units of GAP dehydrogenase; 2.5 mM 3-phosphoglycerate.

Enolase

Enolase was assayed by a modification of the procedure given by Westhead (1966). The reaction mixture contained: 66.7 mM Tris buffer, pH 7.5; 5.0 mM $MgCl_2$; 1.0 mM 2-phosphoglycerate. Due to the presence of nucleic acids, the crude extract was diluted to allow greater sensitivity for detection of phosphoenolpyruvate.

Pyruvate Kinase

Pyruvate kinase was assayed by a modification of the procedure of Valentine and Tanaka (1966). The reaction mixture contained; 66.7 mM Tris buffer pH 7.5; 50.0 mM KCl; 10.0 mM $MgCl_2$; 2.5 mM ADP; 0.25 mM NADH; approximately 6 units of lactic dehydrogenase; 2.5 mM PEP.

NADH Oxidase

NADH oxidase was assayed by a modification of the procedure of von Tigerstrom and Campbell (1966). The reaction mixture contained: 66.7 mM Tris buffer, pH 7.5; 0.25 mM NADH. The cuvette positions were not reversed for this assay so that NADH oxidation was recorded as a decrease in optical density.

NADPH Oxidase

NADPH oxidase was assayed by the same method as NADH oxidase except that 0.25 mM NADPH was substituted for NADH.

Pyridine Nucleotide Transhydrogenase

Pyridine nucleotide transhydrogenase was measured as NAD^+-dependent NADPH oxidation by a procedure similar to that described above. The reaction mixture contained: 66.7 mM Tris buffer, pH 7.5; 0.25 mM NADPH; 0.25 mM NAD^+. The positions of the cuvettes were reversed so that NAD^+-dependent NADPH oxidation was recorded as an increase in optical density. The blank contained no NAD^+.

Histidase

Histidase was assayed in whole cells by a method adapted from Hartwell and Magasanik (1963). Samples of cell suspension were transferred to cold centrifuge tubes and the cells were harvested by centrifugation at 0 C. The pellets were frozen at -20 C until assayed.

Immediately prior to assay, the frozen cell pellets were washed with saline at 0 C and resuspended to the original volume in saline at 0 C. An aliquot of cell suspension was assayed for histidase (formation of urocanate) in a reaction mixture containing 100 mM diethanolamine buffer, pH 9.4, and 10 mM histidine (adjusted to a pH of approximately 9 with KOH).

CHAPTER III

EXPERIMENTAL RESULTS

Isolation of Mutants

Initially, mutants were sought without the benefit of an enrichment procedure. This necessarily required a high frequency, at least 10^{-3}, of the desired mutants since recognition of glucose mutants depended on testing individual colonies. Nečásek, et al. (1967) have reported that prolonged EMS mutagenesis can yield auxotrophs at frequencies exceeding 50 per cent. Survival was quite low but the need for enrichment was overcome. Application of the method to PA-1 yielded a single glucose mutant among approximately 100 colonies from one of 15 segregation tubes examined. It should be noted that under similar conditions of treatment and survival (ca. 10^{-5}), PA-1 did not yield the high auxotroph frequency reported by Nečásek, et al. (1967).

Adelberg et al. (1965) have described conditions for NTG mutagenesis of E. coli permitting over 50 per cent survival with a minimum of one induced mutation per cell. Treatment of PA-1 under the same conditions gave approximately 34 per cent survival and glucose mutants were present in about one half of the segregation tubes at frequencies of about 10^{-3}.

To facilitate the isolation of a large number of glucose mutants, an enrichment procedure was sought. Penicillin has been used with some success in this laboratory, but extremely high concentrations were

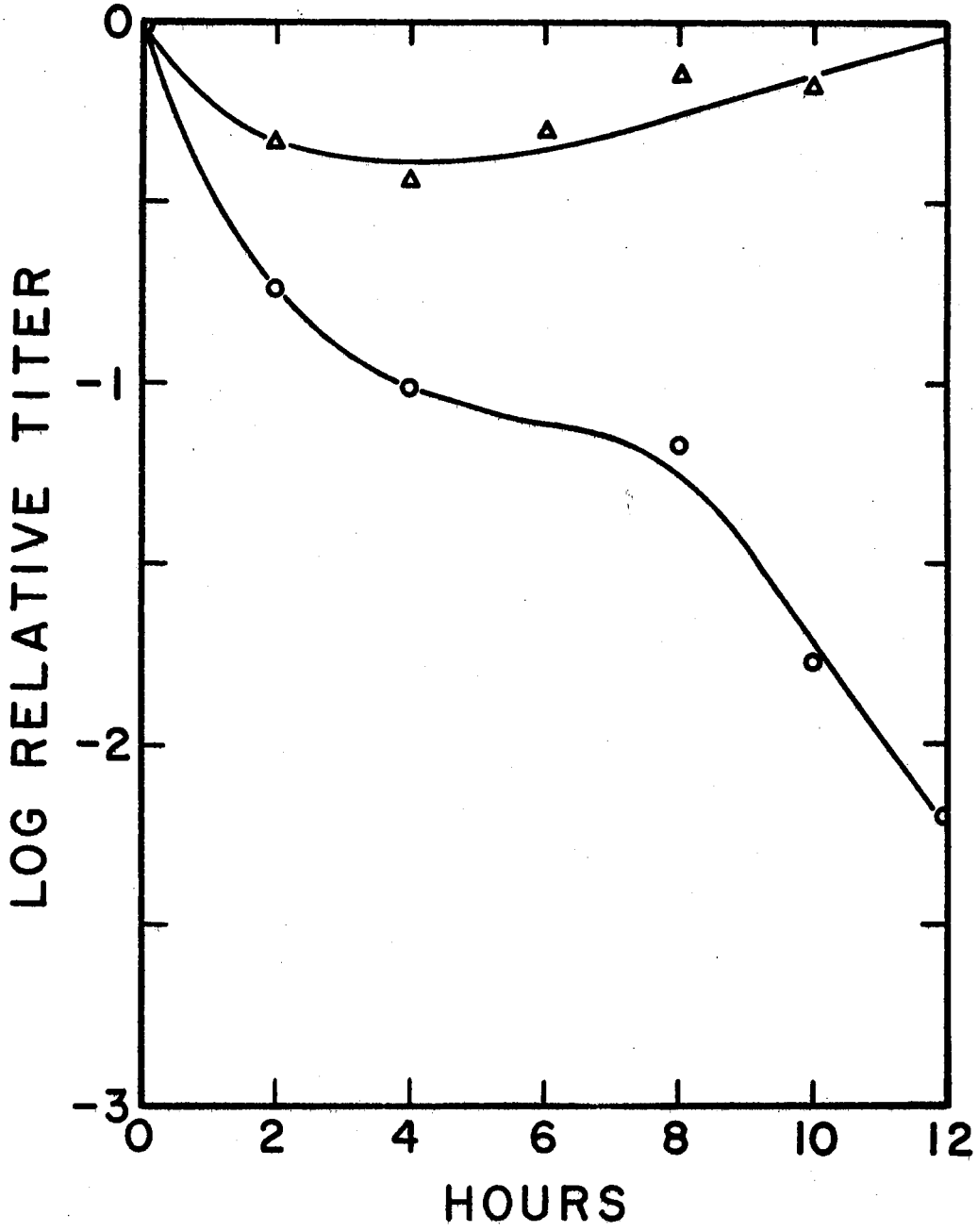
required (Bruce, 1965). Recently Lessie (1969) has used DCS to enrich isoleucine auxotrophs of P. multivorans. The minimum inhibitory concentration of DCS for PA-1 in glucose minimal medium was defined as the lowest concentration which yielded a culture OD less than 0.1 after 20 hours incubation and was found to be approximately 500 µg per ml. NTG-treated cells were enriched in glucose minimal medium containing 500 µg DCS per ml for 6 hours. Glucose mutants were present at frequencies of about 10^{-2} in seven of eight segregation tubes enriched.

In order to evaluate the effectiveness of DCS enrichment, survivals of PA-1 and 707 were compared in glucose minimal medium containing 500 µg per ml DCS. Lactate-grown cells were washed and resuspended to approximately 10^7 cells per ml in 6 ml of DCS-glucose minimal medium. Samples were removed periodically, diluted appropriately, and spread on nutrient agar plates to determine viable counts. The results are shown in Figure 3. Longer enrichments were clearly desirable to increase the killing of wild type relative to mutant cells. The reason for the transient plateau in the wild type curve was unknown but it was reproducible. The slight initial decrease and eventual recovery of viability of the mutant may reflect repairable damage sustained as the cells exhaust their metabolic pools.

Attempts to enrich NTG-mutagenized cells for longer periods of time were not successful. Inevitably the cultures began growing during the extended incubation. This was probably due to an increased frequency of DCS-resistant mutants induced by NTG which grew up during the later stages of enrichment. Ornston et al. (1969) have described a DCS-penicillin enrichment for P. putida and suggested that the simultaneous use of two antibiotics which inhibit cell wall synthesis by

Figure 3. Survival of PA-1 and 707 in Glucose Minimal Medium Containing D-cycloserine.

Lactate-grown cells were washed and resuspended to 10^7 cells per ml in 6 ml of 0.5 per cent glucose minimal medium containing 500 µg per ml DCS. Viable counts were determined at two hour intervals during incubation by spreading appropriately diluted samples on nutrient agar. The logarithm of the relative titer is plotted against time for PA-1 (o) and 707 (Δ).


0
-1
-2
-3
LOG RELATIVE TITER
0
2
4
6
8
10
12
HOURS

supposedly different mechanisms greatly reduces the probability of selecting antibiotic resistant mutants.

Carbenicillin has been used to enrich auxotrophs in this laboratory (Green, 1969). Therefore, a combination of DCS and CAR was expected to prevent growth during extended incubation since mutants resistant to one antibiotic would be killed by the other and double mutants resistant to both antibiotics would be very rare. NTG-treated cells were enriched for 12 hours in glucose minimal medium containing DCS and CAR at final concentrations of 500 and 100 μg per ml, respectively. No growth was apparent at the end of the enrichment. At this point, about 5 per cent of the cells were glucose mutants. However, the frequency of glucose mutants decreased to about 1 per cent during overnight storage of the enriched cells in minimal medium at 4 C.

It should be mentioned that replica plating was much more reliable than direct detection on glucose-yeast extract agar plates. The vast majority of yeast extract-limited colonies emerging from NTG mutagenesis followed by DCS-CAR enrichment were apparently auxotrophs. Increasing the concentration of yeast extract to 0.1 per cent did not eliminate detection of auxotrophs. The frequency of glucose mutants relative to auxotrophs was raised by growing the cells in lactate minimal medium with consequent loss of independence of mutants. Even then, only 5 per cent of the yeast extract-limited colonies were glucose mutants.

During the course of this study, 64 glucose mutants were isolated. On the basis of stability, growth response to various substrates and independence, 17 strains were selected for further study. The origin of these strains is summarized in Table I. Each strain is referred to

TABLE I

DERIVATION OF GLUCOSE MUTANTS

Mutant	Experiment	Separate Segregation	Isolation Procedure		
			Mutagen	Enrichment	Detection
707	1		EMS	none	RP
718	2	+	NTG	none	GY
720	2	+	NTG	none	RP
728	3	+	NTG	DCS	RP
730	3	+	NTG	DCS	RP
732	3	+	NTG	DCS	RP
734	3	+	NTG	DCS	RP
736	3	-	NTG	DCS	RP
738	3	-	NTG	DCS	RP
745	4		NTG	DCS-CAR	GY
747	5	-	NTG	DCS-CAR	RP
748	5	-	NTG	DCS-CAR	RP
755	6	-	NTG	DCS-CAR	RP
757	6	-	NTG	DCS-CAR	RP
760	6	-	NTG	DCS-CAR	RP
773*	7	-	NTG	DCS-CAR	RP
777*	7	-	NTG	DCS-CAR	RP

Experimental details of the isolation procedures are given in Materials and Methods. RP and GY indicate detection by replica plating and glucose-yeast extract agar. Mutants from the same experiment but different segregation tubes are designated by +; those from the same segregation tube are designated by -.

*These strains were enriched and isolated as gluconate mutants.

by its acquisition number. The information in the table should not be interpreted to indicate that DCS enrichment was more efficient than DCS-CAR enrichment. More DCS enriched mutants were investigated since they were known to be independent.

Characterization of Mutants

Growth Studies

Each mutant was tested for its ability to grow on a variety of substrates as sole carbon and energy source by the auxanographic technique (Lederberg, 1946). The cells were grown on nutrient agar slants and washed off with 2 ml of minimal medium. Two drops of cell suspension were added to 10 ml of molten (47 C) minimal agar (1.5 per cent), mixed and poured into a petri dish. Small samples of the substrates were placed at marked points and allowed to soak into the agar. After incubation at 37 C for 18 hours, the plates were scored for growth. The results are summarized in Table II. All the mutants grew on succinate, histidine and allantoin but failed to grow on glucose, gluconate and 2-ketogluconate. This indicated that all the mutants were apparently blocked at a metabolic step after 6-phosphogluconate, the point at which these three pathways converge (cf. Figure 1).

Each mutant was also tested for its ability to grow in glucose, gluconate, glycerol and lactate minimal media. Inocula were prepared as above and 0.1 ml samples of cell suspension were added to 5.9 ml of each medium. The OD of each culture was determined periodically during incubation. Table III summarizes the results. Growth on lactate was comparable to wild type for all mutant strains. Again, all the mutants failed to grow on glucose or gluconate. The ability to grow on

TABLE II

GROWTH OF PA-1 AND GLUCOSE MUTANTS
ON VARIOUS SUBSTRATES

	Growth Medium					
Strain	Glucose	Gluconate	2-Keto-gluconate	Succinate	Histidine	Allantoin
PA-1	+	+	+	+	+	+
707	-	-	-	+	+	+
718	-	±	-	+	+	+
720	-	-	-	+	+	+
728	-	-	-	+	+	+
730	-	-	-	+	+	+
732	-	-	-	+	+	+
734	-	-	-	+	+	+
736	-	-	-	+	+	+
738	-	-	-	+	+	+
745	-	-	-	+	+	+
747	-	-	-	+	+	+
748	-	-	-	+	+	+
755	-	-	-	+	+	+
757	-	-	-	+	+	+
760	-	-	-	+	+	±
773	-	-	-	+	+	±
777	-	-	-	+	+	+

TABLE III

GROWTH OF PA-1 AND GLUCOSE MUTANTS IN VARIOUS MINIMAL MEDIA

Strain	Growth Medium			
	Glucose	Gluconate	Glycerol	Lactate[b]
PA-1	0.921	0.989	0.912	0.782
707	0.046	0.022	0.930	0.770
718	0.080	0.050	0.969	0.782
720	0.032	0.028	0.059	0.803
728	0.037	0.019	0.072	0.789
730	0.026	0.017	0.032	0.789
732	0.028	0.045	0.227 (0.372)[a]	0.796
734	0.024	0.021	0.021	0.846
736	0.021	0.030	0.021	0.903
738	0.029	0.048	0.067	0.810
745	0.025	0.022	0.036	0.776
747	0.027	0.012	0.032	0.796
748	0.030	0.032	0.028	0.824
755	0.024	0.030	0.028	0.789
757	0.027	0.030	0.030	0.673
760	0.028	0.021	0.047	0.886
773	0.021	0.037	0.042	0.878
777	0.048	0.026	0.046	0.903

OD was read after 24 hours incubation except as noted.
[a]OD was read after 36 hours incubation.
[b]OD was read after 12 hours incubation.

glycerol was variable. Glycerol-grown mutants were subcultured into glucose minimal medium. In each case the glycerol-grown cells failed to grow on glucose indicating that growth on glycerol was not due to reversion. Therefore, growth response to glycerol provided a useful criterion for grouping the mutants.

The fact that most of the glucose mutants were unable to grow on glycerol could be explained in several ways. NTG mutagenesis was reported to induce an unusually high frequency of double mutants (Adelberg et al., 1965). This explanation was considered unlikely since glycerol-negative glucose mutants were so frequent and glycerol was an unselected marker. If glucose and glycerol genes were closely linked, a deletion could inactivate both genes. However, most of the strains were observed to revert, indicating that the defect was not a deletion. Alternatively, growth on glycerol might depend on an early step in glucose catabolism (i.e., prior to KDPG cleavage) which was defective in most of the glucose mutants. This seemed improbable since an obvious dependence of glycerol dissimilation on early glucose enzymes was not suggested by the known pathways (cf. Figure 1). Glycerol and glucose catabolism converge at glyceraldehyde 3-phosphate. Glycerol-negative glucose mutants could lack pyruvate kinase, the only nonamphibolic activity in the conversion of triose phosphate to pyruvate. It should be noted that this interpretation requires an additional assumption. KDPG cleavage yields pyruvate directly; therefore, failure to grow on glucose and related compounds must be assumed to be due to inhibition by intermediates accumulated from triose phosphate.

Glucose is generally quite effective in eliciting catabolite repression of other catabolic pathways. An intermediate derived from

glucose, rather than glucose itself, is thought to effect the repression (Magasanik, 1961). Lessie and Neidhardt (1967b) have shown that several enzymes of histidine degradation are subject to catabolite repression by glucose in P. aeruginosa. Therefore, different glucose mutants should accumulate different intermediates which might be expected to affect growth on histidine differently. Inocula were prepared as above and 0.1 ml samples of cell suspension were subcultured into 5.9 ml of minimal medium containing 0.25 per cent glucose, 0.25 per cent histidine or 0.25 per cent histidine plus 0.25 per cent glucose. The OD of each culture was determined periodically during incubation. The results are given in Table IV. All strains tested were inhibited to some extent. Of the glycerol positive strains, 718 and 732 were inhibited less than was 707. Among the glycerol negative strains tested, 734 and 736 were inhibited the least. Severe inhibition was observed with 720, 728 and 730.

Each mutant was also tested for inhibition by glucose of growth on nutrient broth. Inocula were prepared as above and 0.1 ml samples were subcultured into 5.9 ml of nutrient broth and nutrient broth containing 0.5 per cent glucose. The OD of each culture was determined periodically during incubation. Table V summarizes the results. Strains 720, 745, 747, 748, 755 and 757 suffered a severe inhibition which was followed by lysis in four of the six strains. This response was considered to be a group characteristic and therefore provided a criterion for subgrouping the glycerol negative glucose mutants. Strains 732 and 736 showed essentially no inhibition due to glucose. All other glucose mutants exhibited inhibition to various degrees.

TABLE IV

INHIBITION OF GROWTH ON HISTIDINE
BY GLUCOSE

Mutant	Growth Medium			
	Glucose[a]	Histidine[b]	Histidine plus	Glucose[c]
707	0.022	0.745	0.104	0.340
718	0.045	0.721	0.412	0.817
720	0.025	0.763	0.042	0.052
728	0.018	0.745	0.062	0.132
730	0.021	0.704	0.040	0.072
732	0.027	0.727	0.367	0.727
734	0.021	0.710	0.118	0.683
736	0.014	0.745	0.112	0.673

[a]OD was read after 20 hours incubation.
[b]OD was read after 12 hours incubation.
[c]OD was read after 12 and 20 hours incubation (third and fourth columns respectively).

TABLE V

INHIBITION OF GROWTH ON NUTRIENT BROTH BY GLUCOSE

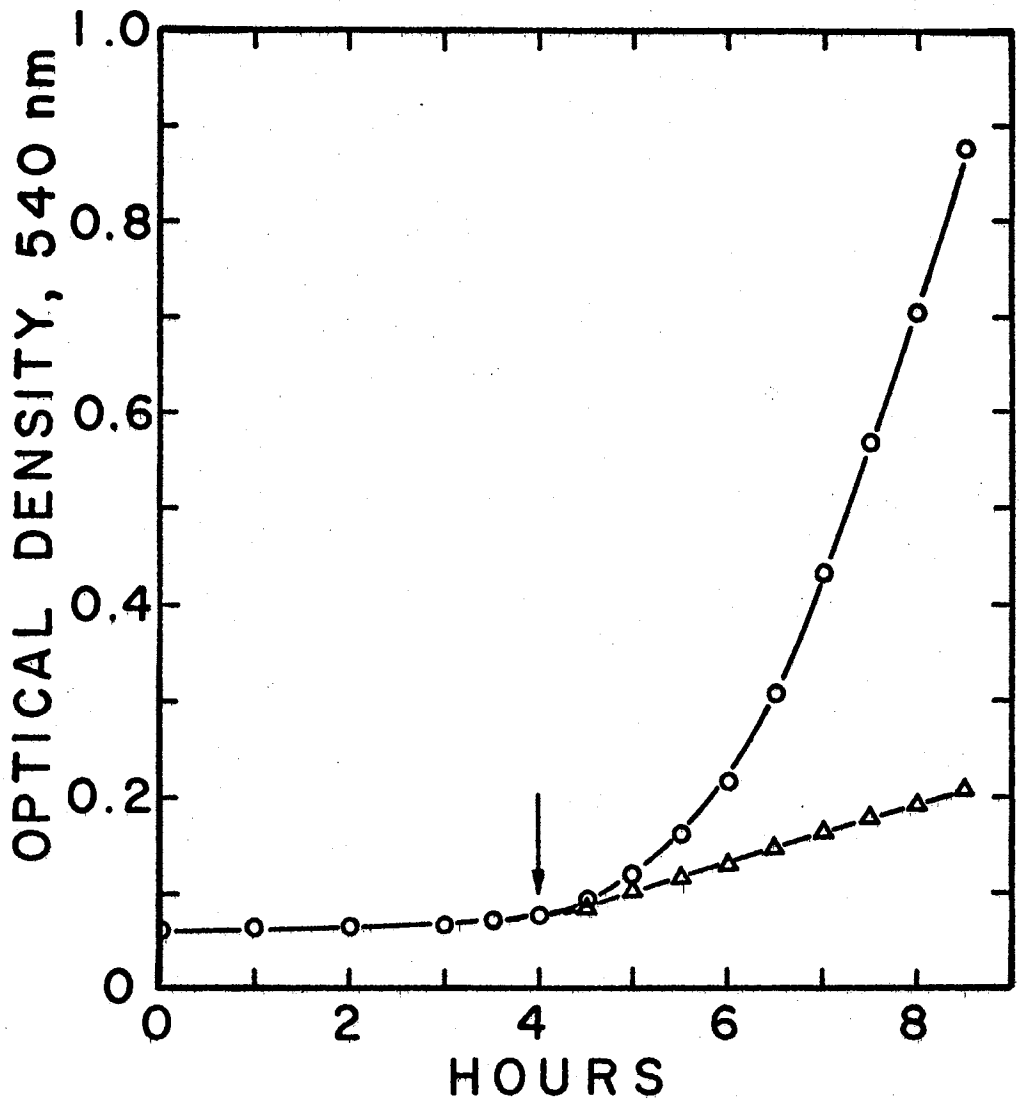
Strain	Growth Medium		
	Nutrient Broth	Nutrient Broth Plus Glucose	
	OD, 8 hr.	OD, 4 hr.	OD, 8 hr.
PA-1	0.782		1.02
707	0.824		0.721
718	0.789		0.862
720	0.757	0.156	0.090
728	0.763		0.581
730	0.663		0.538
732	0.803		0.683
734	0.699		0.478
736	0.727		0.699
738	0.673		0.344
745	0.710	0.115	0.121
747	0.733	0.137	0.104
748	0.624	0.046	0.078
755	0.716	0.156	0.115
757	0.721	0.199	0.146
760	0.710		0.488
773	0.683		0.354
777	0.789		0.538

Several enzymes of the histidine catabolic pathway of P. aeruginosa have been shown to be inducible (Lessie and Neidhardt, 1967b). In the previous experiment, uninduced cells were subcultured into a mixture of histidine and glucose. Under these conditions, the observed growth inhibition could be due to interference with the induction process as well as catabolite repression. In order to avoid any effect of glucose on induction, glucose was added to a culture which was induced and just beginning to grow on histidine. Strain 707 was grown in nutrient broth, washed and subcultured into 25 ml of 0.25 per cent histidine minimal medium in each of two 250 ml side arm flasks. After the cultures began to grow, glucose was added to the experimental flask to a final concentration of 0.25 per cent and OD was determined periodically for each culture during incubation. The results of a typical experiment are given in Figure 4. Growth of 707 after addition of glucose was linear. The rate of linear growth depended on the point at which glucose was added, later addition yielding higher rates. The linearity of growth after addition of glucose was interpreted as resulting from dilution of an essential, growth limiting substance by cell division. A logical choice for such a substance was one (or more) of the histidine catabolic enzymes which must provide carbon and energy for growth. Therefore, linear growth was thought to reflect catabolite repression elicited by glucose or its intermediates.

Nutrient broth-grown 707 cells converted glucose to an intermediate tentatively identified as gluconate which appeared in the medium. The data supporting these statements are presented in the next section. The effect of this excreted intermediate on growth of uninduced 707 on histidine was examined.

Figure 4. Effect of Glucose on 707 Growing on Histidine.

Nutrient broth-grown cells were washed and resuspended in 25 ml of 0.25 per cent histidine minimal medium in each of two 250 ml side arm flasks. Glucose was added to the second flask to a final concentration of 0.25 per cent at the time indicated by the arrow. Growth on histidine (o); growth on histidine after addition of glucose (Δ).


OPTICAL DENSITY, 540 nm
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
HOURS

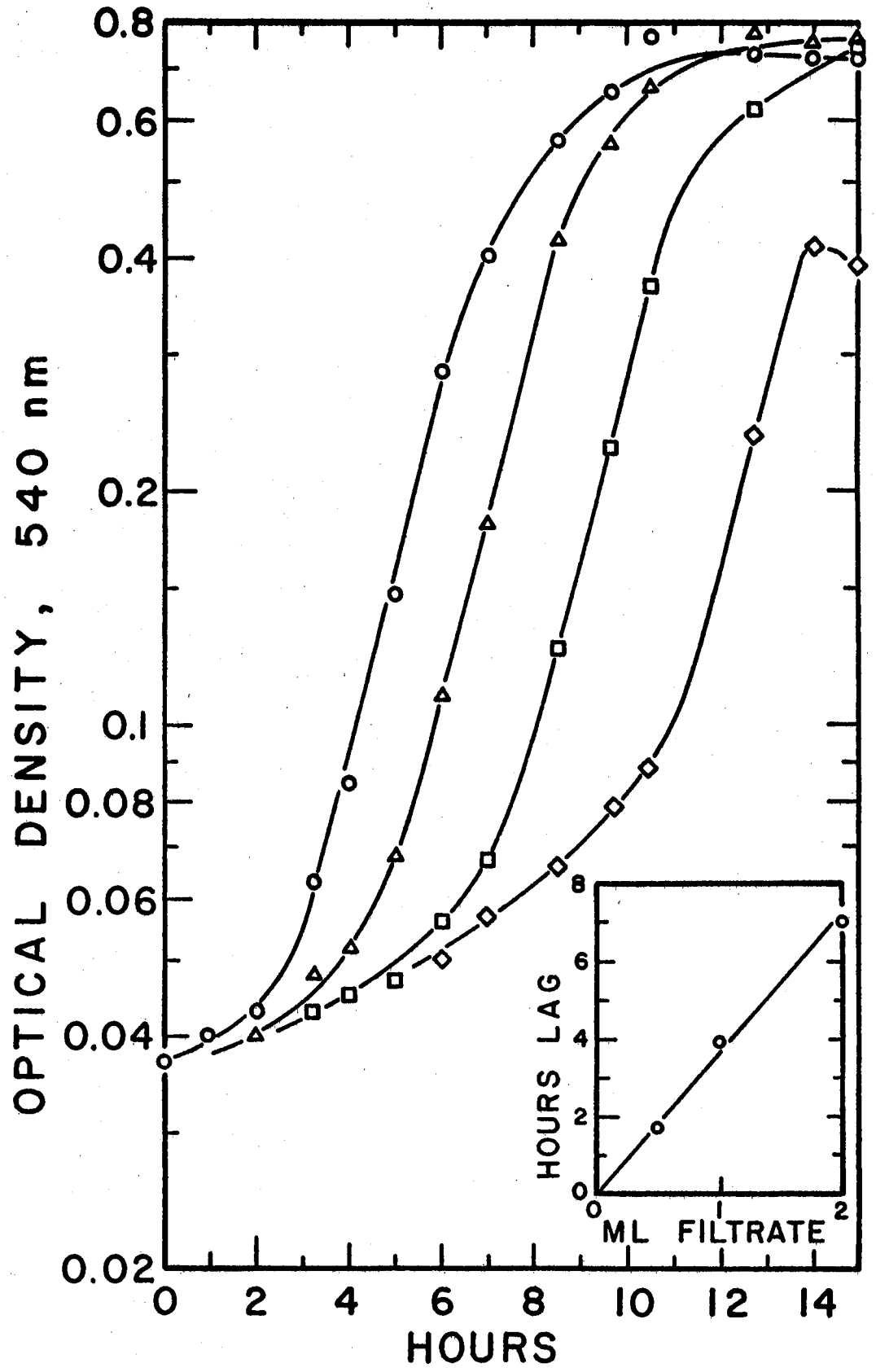
The intermediate was prepared by exposing washed nutrient broth grown 707 cells in 0.02 M TES buffer, pH 7.0, to 0.2 mg per ml glucose. When the glucose was exhausted, as determined by glucostat assay, the cells and medium were separated by Millipore filtration (HA, 0.45 μm pore size). The filtrate was used directly as the source of excreted intermediate; this corresponded to a 0.02 per cent solution of the intermediate, assuming complete recovery.

A series of tubes containing 0.25 per cent histidine minimal medium was prepared with 0.0, 0.5, 1.0, and 2.0 ml of filtrate, respectively. Each tube contained TES buffer at a final concentration of 0.01 M and one half the usual minimal medium salts concentration. The tubes were inoculated with 0.1 ml aliquots of washed, nutrient broth-grown (i.e., uninduced) 707 cell suspension. The OD of each culture was determined periodically during incubation. Figure 5 presents the data as semi-logarithmic plots. The filtrate delayed attainment of exponential growth at the control rate. The length of this lag period was proportional to the amount of filtrate present as shown in the inset of Figure 5. This indicated that at low concentration the effect of the intermediate was apparently on the induction process and could be overcome, perhaps by further metabolism to an inactive form.

If the excreted intermediate were metabolized to an inactive form, filtrate which had exerted its effect (i.e., challenged filtrate) should be unable to effect a growth lag. To test this possibility, equivalent amounts of challenged and unchallenged filtrate were compared for their ability to produce a growth lag.

Figure 5. Effect of Accumulated Glucose Catabolite on Growth of 707 on Histidine.

Nutrient broth-grown cells were washed and resuspended in 6 ml of 0.25 per cent histidine minimal medium containing one half the usual salts concentration, 0.01 M TES buffer, pH 7.0, and the following amounts of filtrate: (1) 0 ml; (2) 0.5 ml; (3) 1 ml; (4) 2 ml. The filtrate was prepared as described in the text. Growth in the absence of filtrate (o); growth in the presence of 0.5 ml filtrate ($\triangle$); growth in the presence of 1 ml filtrate ($\square$); and growth in the presence of 2 ml filtrate ($\diamond$). The inset shows the relation between growth lag and amount of filtrate.


OPTICAL DENSITY, 540 nm
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0
2
4
6
8
10
12
14
HOURS
HOURS LAG
8
6
4
2
0
0
2
ML FILTRATE

Challenged filtrate was prepared by repeating the above growth experiment with 2 ml of filtrate (cf. Figure 5). Growth occurred after the expected lag period. When growth was essentially complete, the cells and medium were separated by centrifugation and the supernatant was filtered to remove any residual cells. This filtrate was used directly as challenged filtrate. Based on dilution, 3 ml of challenged filtrate were equivalent to 1 ml of unchallenged filtrate.

Three tubes of 0.25 per cent histidine minimal medium containing no addition, 3 ml of challenged filtrate and 1 ml of unchallenged filtrate, respectively, were prepared as before. Each tube was inoculated with 0.1 ml of washed, nutrient broth-grown (i.e., uninduced) 707 cell suspension and OD was read periodically during incubation for each culture. Semi-logarithmic plots of the data are given in Figure 6. Unchallenged filtrate produced approximately the predicted lag. Unexpectedly, challenged filtrate hastened the attainment of exponential growth at the control rate. A factor which stimulated the induction process must have accumulated in the medium during the preparation of challenged filtrate. Apparently the factor was formed as the cells grew on histidine. Therefore, the lag produced by unchallenged filtrate did not necessarily represent the time required to metabolize the intermediate to an inactive form.

The effect of gluconate on growth of induced and uninduced 707 on histidine was also examined. The experimental procedure was the same as described previously for glucose except that the cultures were grown in tubes rather than flasks and a tube containing histidine plus gluconate was also included. The results are shown in Figure 7. Addition of gluconate to induced cells caused linear growth indicating that

Figure 6. Effect of Challenged Filtrate on Growth of 707 on Histidine.

Nutrient broth-grown cells were washed and resuspended in 6 ml of 0.25 per cent histidine minimal medium containing one half the usual salts concentration, 0.01 M TES buffer, pH 7.0, and the following additions: (1) no addition; (2) 3 ml challenged filtrate; (3) 1 ml filtrate. Challenged filtrate was prepared as described in the text. Growth in the absence of filtrate and challenged filtrate (o); growth in the presence of 3 ml challenged filtrate (△) and 1 ml filtrate (□).

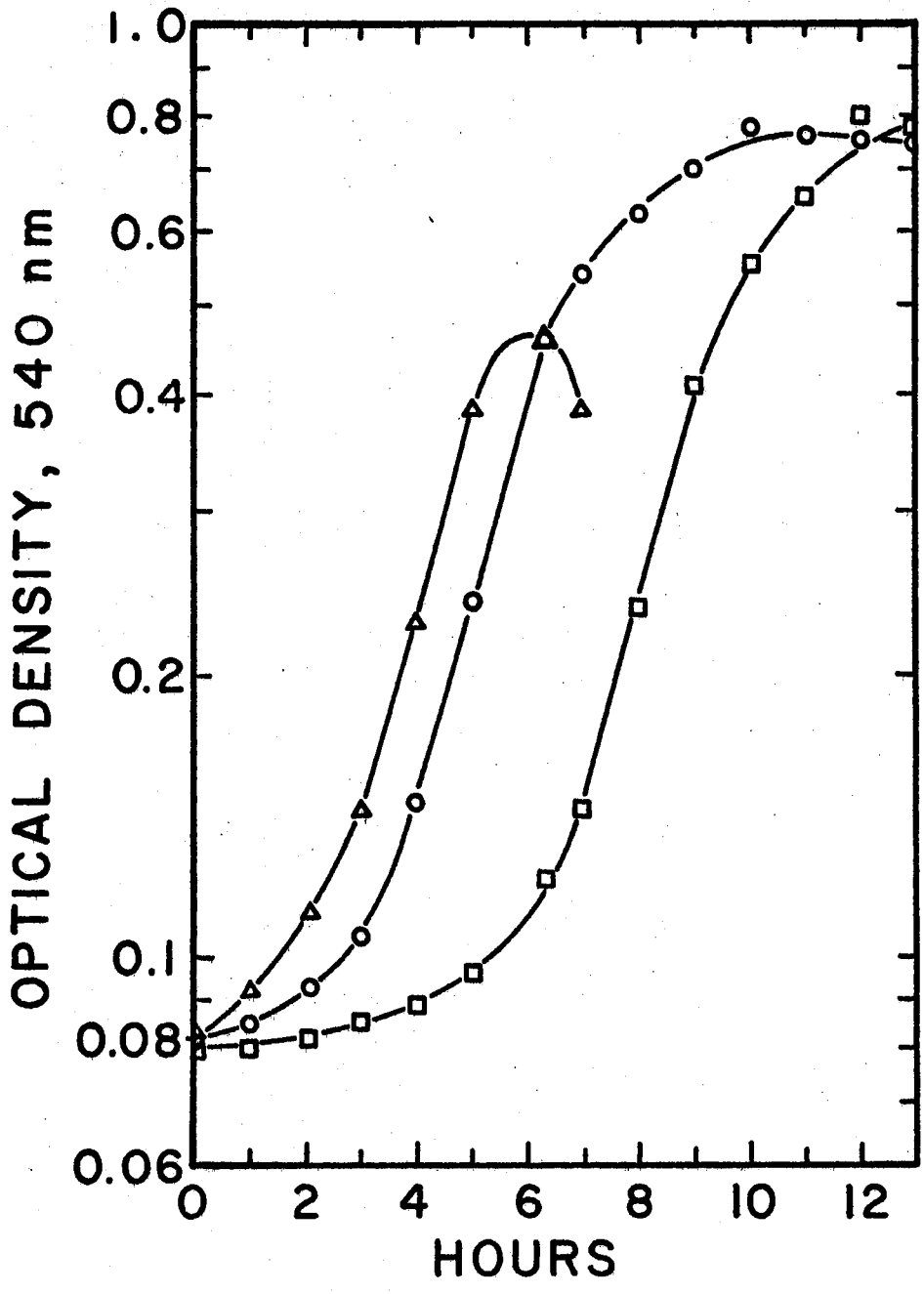
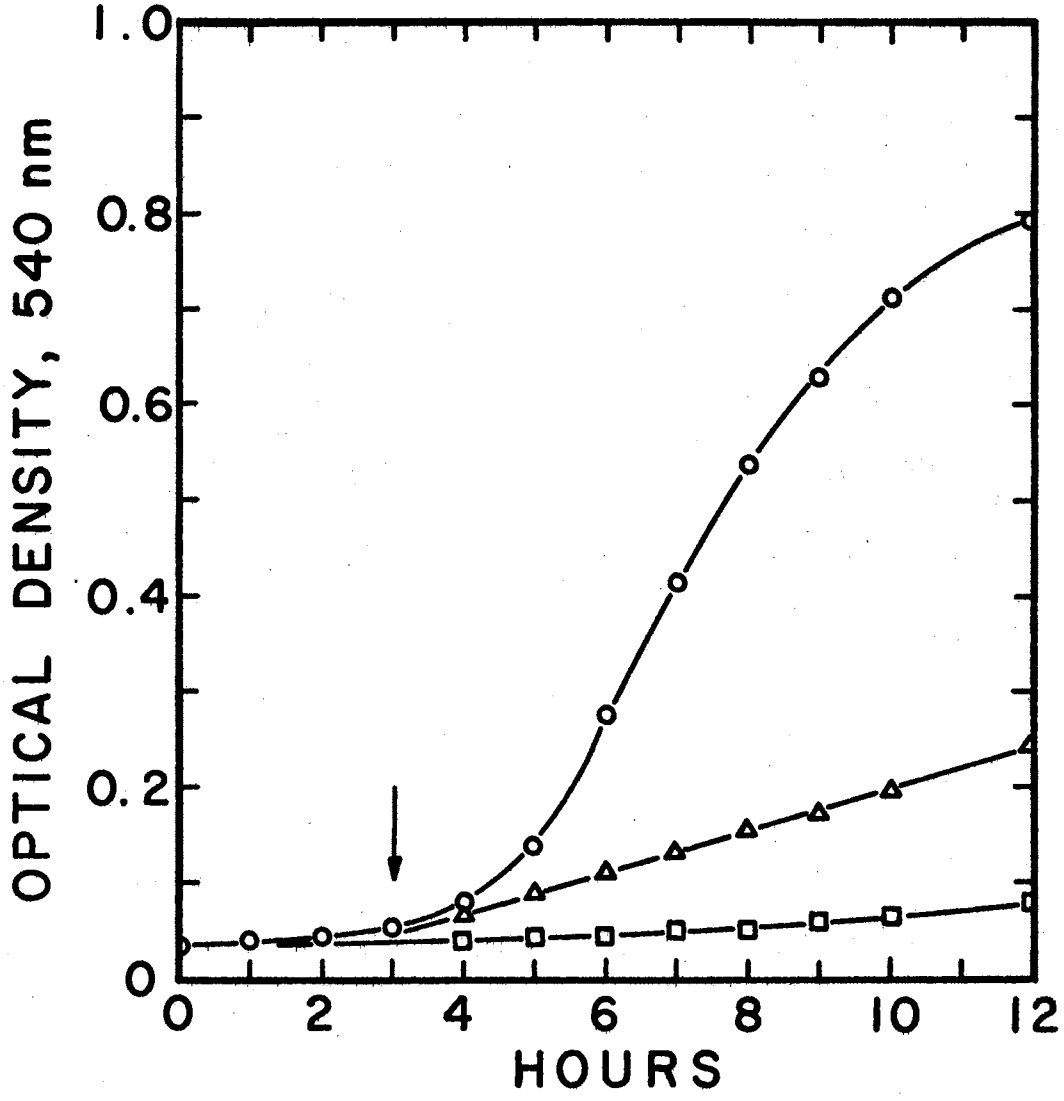

OPTICAL DENSITY, 540 nm
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0
2
4
6
8
10
12
HOURS

Figure 7. Effect of Gluconate on Growth of 707 on Histidine.

Nutrient broth-grown cells were washed and resuspended in 6 ml of each of the following minimal media; (1) 0.25 per cent histidine, in duplicate; (2) 0.25 per cent histidine plus 0.25 per cent gluconate. Gluconate was added to the second histidine tube at the time indicated by the arrow. Growth on histidine (o); growth on histidine after addition of gluconate (Δ); growth on histidine plus gluconate (□).


OPTICAL DENSITY, 540 nm
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
HOURS

glucose itself was not essential for this effect and that gluconate or other intermediates derivable from glucose and gluconate were responsible. Like glucose, gluconate caused severe growth inhibition of uninduced cells as predicted by the filtrate effect. The slower growth of the histidine control culture compared to that of the analogous glucose experiment was undoubtedly due to poorer aeration of tube cultures compared to flask cultures.

Catabolite repression of histidine degrading enzymes has been shown to be relieved in A. aerogenes and P. aeruginosa when histidine served as the sole nitrogen source (Neidhardt and Magasanik, 1957; Lessie and Neidhardt, 1967b). The type of experiment described above was repeated with NH_4Cl omitted from all media. Histidine was the sole nitrogen source. Growth on histidine after addition of glucose was linear as before. The apparent catabolite repression was not relieved when histidine was sole nitrogen source. Likewise, growth inhibition of uninduced cells was not altered when histidine was sole nitrogen source.

Glucose Metabolism and Transport

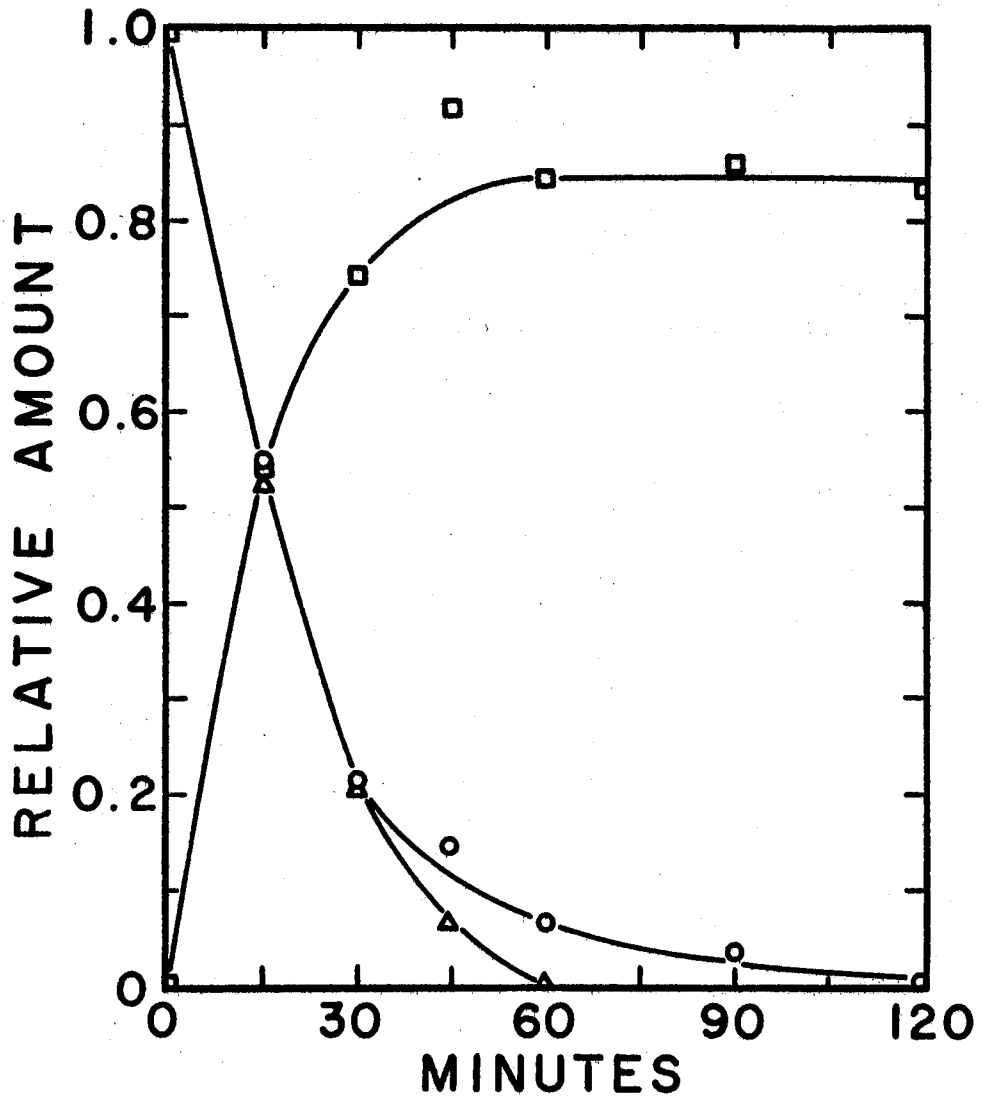
Preliminary experiments with 707 demonstrated that nutrient broth-grown cells catalyzed the disappearance of glucose from the medium as determined by glucostat assay. The fate of uniformly labeled ^{14}C-glucose was examined to clarify the extent of metabolism and identify any intermediates which might accumulate. Strain 707 was grown on nutrient broth, washed and resuspended in minimal medium. Three 250 ml flasks, each containing 25 ml of cell suspension (OD, 0.561), were prepared. The flasks were equilibrated to 37 C in a water bath shaker.

At zero time, glucose was added to each flask to a final concentration of 0.2 mg per ml (0.2 μc per mg). Cells and medium were separated by Millipore filtration (HA, 0.45 μm pore size) of 5 ml samples periodically during incubation. Three sequential samples were removed from each flask in series. Glucose remaining in 1 ml aliquots of filtrate was determined by the glucostat assay. Each filtrate was also analyzed chromatographically. Samples of 0.1 ml were spotted on strips of Whatman No. 4 chromatography paper. The chromatograms were developed by the descending technique with n-butanol: pyridine: water (6:4:3). The strips were then counted on a Nuclear-Chicago Actinograph III strip counter. Peak areas were determined by planimetry and used directly as a measure of activity in arbitrary units. The results are summarized in Figure 8. All data have been plotted as relative values to facilitate comparison. Glucose chromatographed with an R_f of 0.40 in this system. Measurements of the disappearance of glucose from the medium by glucostat assay and as activity chromatographing with an R_f of 0.40 were in good agreement. A single peak with an R_f of 0.10 appeared in the medium, coincident with the disappearance of glucose. The appearance of this material was plotted relative to the initial glucose activity. By 60 minutes, essentially all of the glucose had been consumed and 85 per cent of its activity had reappeared in the medium as an excreted intermediate. The intermediate remained at the same concentration for at least the next 60 minutes.

The excreted intermediate was tentatively identified as gluconate based on the following information. Authentic gluconate was detected by an alkaline $AgNO_3$ acetone dip procedure (Smith, 1960). The authentic gluconate chromatographed as a relatively long streak with an R_f

Figure 8. Conversion of Glucose to Gluconate by 707.

Nutrient broth-grown cells were washed and resuspended to an optical density of 0.561 in minimal medium. At zero time, U-^{14}C-glucose was added to a final concentration of 0.2 mg per ml (0.2 μc per mg). Cells and medium were separated by Millipore filtration of 5 ml samples withdrawn at intervals. The relative amounts of glucose remaining as determined by glucostat (o) and activity chromatographing with R_f 0.40 (Δ) are plotted against time. The appearance of gluconate (activity chromatographing with R_f 0.10) relative to the initial glucose activity is also plotted against time (□).


1.0
0.8
0.6
0.4
0.2
0
RELATIVE AMOUNT
0
30
60
90
120
MINUTES

of 0.10. Only the leading edge of the unknown spot could be detected chemically since a salt from the minimal medium overlapped and interfered with detection.

A variety of glucose mutants were tested for their ability to remove glucose from the medium. Tiwari and Campbell (1969) and Phibbs and Eagon (1970) have cited unpublished experiments which demonstrate an inducible glucose transport system in P. aeruginosa. Therefore, each strain was grown in 6 ml of nutrient broth containing 0.5 per cent glucose to induce whatever glucose metabolic capabilities the mutants may retain. Resting cell suspensions were prepared by washing and resuspending the cells to volume in nitrogen-free minimal medium. Glucose was added to a final concentration of 0.2 mg per ml and the resting cell suspensions were incubated on a reciprocal shaker at 37 C for 2 hours. Cells and medium were separated by centrifugation and 1 ml aliquots of supernatant were assayed for residual glucose by the glucostat assay. The percentage of glucose removed by each strain is given in Table VI. Only 736 failed to remove glucose from the medium. As noted in Table V, 720 exhibited the lysis phenomenon in nutrient broth containing 0.5 per cent glucose. Fortuitously, the cells used in this experiment were harvested before lysis occurred.

In order to confirm that 736 was unable to transport glucose, an uptake experiment was performed. The accumulation of glucose-U-^{14}C by PA-1 and 736 cells was compared. Each strain was grown in nutrient broth containing 0.5 per cent glucose, washed and resuspended in minimal medium. The cell suspension was adjusted to an OD of 0.347 and 25 ml in a 250 ml flask were equilibrated to 37 C in a water bath shaker. Glucose was added to a final concentration of 0.05 mg per ml

TABLE VI

REMOVAL OF GLUCOSE BY INDUCED RESTING CELL SUSPENSIONS

Strain	Per Cent Glucose Removed
PA-1	97
707	96
720	92
730	90
734	94
736	0
773	88
777	78

Glucose remaining in the medium was measured by the glucostat assay after 2 hr. incubation.

(0.2 μc/mg) and 1 ml samples were removed at 2 minute intervals with a syringe. The samples were ejected onto prewetted Millipore filters and washed twice with 1 ml of cold minimal medium. The filters were placed in scintillation vials and dried in a stream of hot air. The filters were then crushed and 10 ml of ethanol-toluene scintillation fluid were added. The scintillation fluid contained 4 g of 2,5-diphenyloxazole (PPO) and 0.2 g of 1,4-bis [2-(5-phenyloxazolyl) benzene] (POPOP) per liter of ethanol-toluene (4:6). The samples were counted with a Nuclear-Chicago liquid scintillation counter, Model 720. The results are presented in Figure 9 as cpm corrected for background. Strain 736 failed to accumulate glucose and was therefore considered to be a transport mutant.

Genetic Analysis

The mutants were grouped on a genetic basis by qualitative transduction tests with bacteriophage F116. A modification of the spot plate method of Murphy and Rosenblum (1964) was used as described in Materials and Methods. Each mutant was crossed with the wild type and all other mutants. Transductants were selected on glucose minimal medium. The results were recorded as the number of colonies appearing within the area of the phage spot and are presented in Table VII. These numbers are not recombination frequencies. Regions of the plate not spotted with phage served as a control for reversion of the recipient. Strain 732 reverted at high frequency. The results of crosses with 732 as recipient were scored + if growth occurred within the phage spot and are included because phage propagated on 718 or 732 produced clear areas indicating a close relationship between these strains.

Figure 9. Accumulation of Activity From Glucose-U-^{14}C by PA-1 and 736.

Cells grown on nutrient broth containing 0.5 per cent glucose were washed and resuspended to an optical density of 0.347 in minimal medium. At zero time, glucose was added to a final concentration of 0.05 mg per ml (0.2 µc per mg). At two minute intervals, 1 ml samples were removed and the cells were collected on Millipore filters and washed twice with 1 ml of cold minimal medium. The filters were transferred to scintillation vials, dried and counted in a liquid scintillation counter. The results are given as cpm corrected for background. Accumulation of activity by PA-1 (o) and 736 (Δ).
Note: Counts were not corrected for retention by the filter, which amounted to approximately 50 cpm, determined as the average of 5 measurements.

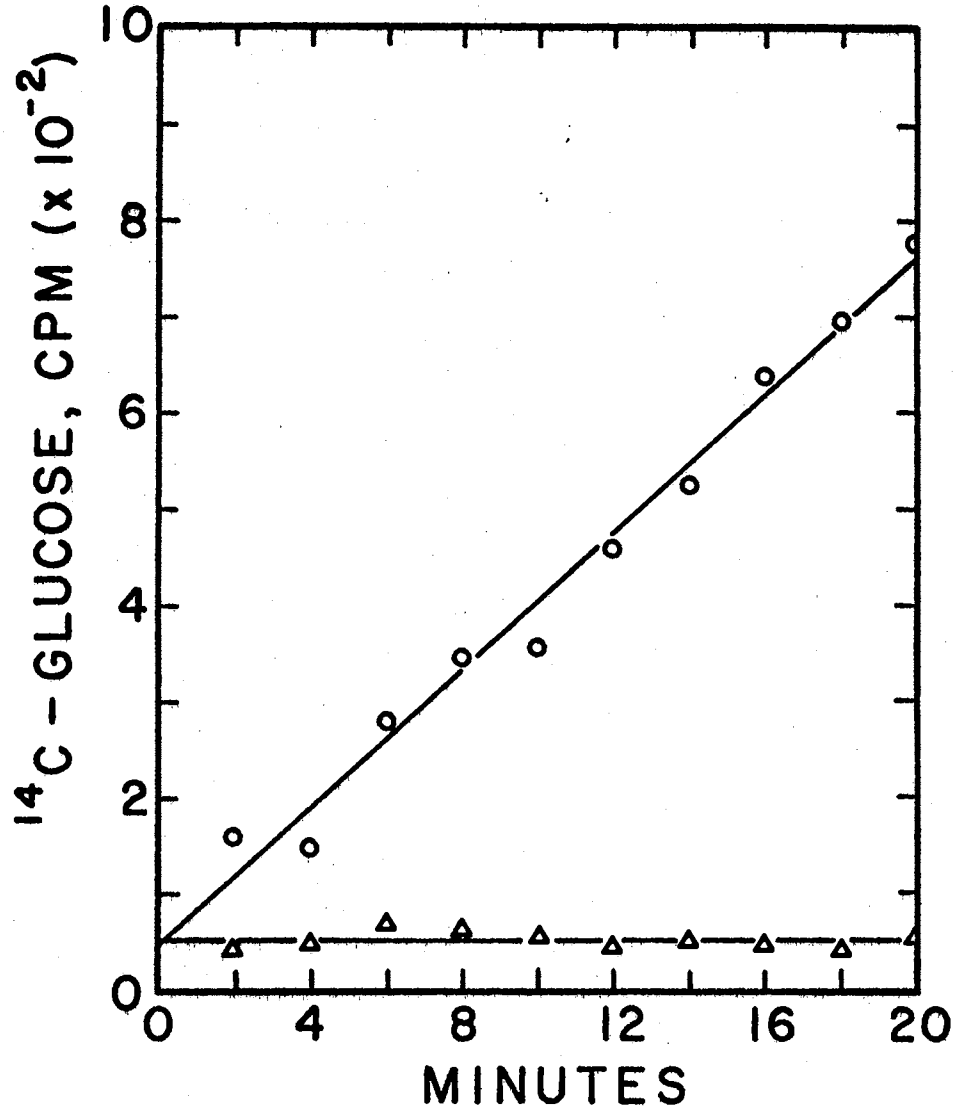

^{14}C - GLUCOSE, CPM (x 10^{-2})
10
8
6
4
2
0
0
4
8
12
16
20
MINUTES

TABLE VII

TRANSDUCTION BETWEEN GLUCOSE MUTANTS

	Recipient																
Donor	707	718	732	728	760	773	777	734	738	730	736	720	745	747	748	755	757
707	0	2	+	0	0	0	0	0	0	0	0	1	0	2	1	0	0
718	1	0	0	1	0	0	1	2	2	0	0	12	4	8	1	20	2
732	2	0	0	0	0	0	0	0	0	2	2	2	2	0	2	3	0
728	2	2	+	0	0	0	0	0	0	1	1	2	3	2	2	1	1
760	0	0	+	0	0	0	0	0	0	0	0	0	0	0	1	1	4
773	1	1	+	1	2	0	0	2	1	0	0	15	10	15	10	16	4
777	2	2	+	1	1	1	0	0	0	1	0	10	19	15	14	16	7
734	0	2	+	1	0	0	0	0	0	0	0	1	1	0	0	1	0
738	0	2	+	0	0	0	0	0	0	0	0	2	0	2	3	0	1
730	0	2	+	0	0	0	0	0	0	0	0	2	7	2	0	2	0
736	1	1	+	0	1	0	0	0	0	0	0	3	6	11	4	5	3
720	0	2	+	1	0	+	0	1	0	0	1	0	0	0	0	0	0
745	4	2	+	1	3	1	2	3	1	3	2	1	0	0	0	0	0
747	3	4	+	2	3	0	2	5	0	0	0	0	0	0	0	0	0
748	19	24	+	13	23	9	18	18	15	20	14	1	1	0	0	0	0
755	1	2	+	1	3	0	0	4	2	1	2	1	0	0	0	0	0
757	1	4	+	2	4	0	0	2	2	0	1	0	1	0	0	0	0
PA-1	8	10	+	6	14	5	9	16	5	8	12	2	4	3	6	5	2

Transduction tests clearly separated the mutants into two major groups which are enclosed in solid lines in Table VII. This separation corresponded exactly to those strains which did and did not lyse in glucose-supplemented nutrient broth (cf. Table V). Mutants 707, 718, 732, 728, 760, 773, 777, 734, 738, 730 and 736 (group I) did not lyse, whereas mutants 720, 745, 747, 748, 755 and 757 (group II) did lyse.

Group I was known to be heterogeneous on the basis of growth and glucose removal studies. Mutants 707, 718 and 732 were able to grow on glycerol. This subgroup is enclosed in broken lines in the table. The remainder of group I could be further subdivided on the basis of enzyme profiles for each strain. These subgroups are also outlined in broken lines in the table. The detailed enzyme data supporting these subdivisions is presented in a later section. The subgroup containing strains 730 and 736 could be further subdivided since 730 was permeable to glucose but 736 was not.

These data suggested that the mutations represented by group I must be relatively distant on the chromosome from the mutations represented by group II. Mutants of group I were generally as good as or better than PA-1 as donor with mutants of group II as recipients. This was supported by the reciprocal crosses although the numbers were slightly lower.

As discussed previously, many of the glucose mutants were also unable to grow on glycerol. If failure to grow on both media were due to the same lesion, revertants could be selected on either glucose or glycerol and would be able to grow on the unselected medium. Alternatively, if these strains were actually double mutants, revertants selected on one medium would not be expected to grow on the other medium.

Spontaneous revertants were isolated by spreading about 5×10^8 cells on either glucose or glycerol minimal medium. Revertants were also induced by NTG mutagenesis of nutrient broth-grown cells. Cells surviving mutagenesis were regrown in nutrient broth and spread on both media as above. Colonies appearing on the plates were spot tested on both media. Representative strains were retested on both media for confirmation. As a check to insure that the revertants were derived from the glycerol-negative glucose mutants and were not contaminants, about one half of the confirmed revertants were tested for sensitivity to phage PØ-6 by cross streaking cells on phage as follows. Nutrient agar plates were supplemented with NaCl by spreading 0.2 ml of sterile saline on the surface. Phage were then applied as a line with a sterile cotton swab soaked in phage suspension (titer ca. 10^9 pfu per ml). Cell samples were drawn across the phage streak with sterile wooden applicators. After incubation at 37 C for about 12 hours, sensitive strains showed no growth beyond the phage streak.

Table VIII summarizes the results of the reversion analysis. Type I revertants grew only on the selected substrate; Type II revertants grew on both the selected and unselected substrate. It must be emphasized that the entries in the table are merely confirmed, phage-sensitive representatives of the revertant types. The fact that a given representative was spontaneous (S) does not necessarily mean that other NTG induced (N) representatives were not or could not have been isolated and vice versa.

Previously, several possible explanations for the unexpected high proportion of glycerol-negative glucose mutants were offered. The reversion analysis eliminated several of these. Strain 736 was

TABLE VIII

TYPES OF REVERTANTS SELECTED ON GLUCOSE AND GLYCEROL MINIMAL MEDIA

	Selection Medium			
	Glucose		Glycerol	
Mutant	Type I	Type II	Type I	Type II
734	-	N	S,N	S
736	-	-	N	-
738	-	N	S	N
755	-	S,N	N	N
760	-	N	S	S,N
773	-	S,N	S	S
777	-	S,N	S	S,N

Type I revertants grew only on the selection medium; Type II revertants grew on both media. Entries of S (spontaneous) and/or N (NTG-induced) indicate that at least one revertant of this type was confirmed and phage-sensitive. Minus indicates that no revertant of this type was observed.

somewhat exceptional and will be considered separately. The fact that revertants could be isolated argued against deletions as the cause of the double defect. All revertants selected on glucose were Type II indicating that failure to grow on both glucose and glycerol was due to the same lesion. This was supported by the fact that Type II revertants selected on glycerol were found for each strain (except 736). Interestingly, Type I revertants selected on glycerol were also obtained for all strains including 736. This paradoxical fact made it highly unlikely that the lesion common to both glucose and glycerol catabolism was pyruvate kinase as restoration of function should not be substrate specific. However, the remaining possibility that glycerol catabolism depends in some obscure manner on the initial steps of glucose catabolism suffered the same criticism. Nevertheless, Type I revertants selected on glycerol represented mutational acquisition of an alternate mechanism for circumventing the original dysfunction in a substrate specific manner.

Strain 736 failed to yield either spontaneous or NTG induced glucose or Type II glycerol revertants. Furthermore, it had never been observed to revert at any time since its isolation. Therefore, 736 was considered to be a deletion mutant. Apparently the glycerol specific mode of reversion was not included in the deletion since Type I revertants were obtained on glycerol.

Enzymological Analysis

P. aeruginosa degrades glucose and related compounds via the Entner-Doudoroff (ED) and to a lesser extent the hexose monophosphate (HMP) pathways (Wang et al., 1959). The lack of a functional

Embden-Meyerhof-Parnas (EMP) pathway has been traced to two different enzyme defects in two different strains of P. aeruginosa. Lessie and Neidhardt (1967a) failed to detect fructose diphosphate aldolase in cell free extracts of P. aeruginosa ATCC 7700. No other EMP enzyme activities were reported. Tiwari and Campbell (1969) found that phosphofructokinase was the only EMP enzyme missing in P. aeruginosa ATCC 9027. Although both defects resulted in a catabolically nonfunctional EMP pathway, Campbell's P. aeruginosa ATCC 9027 could convert triose phosphate to hexose phosphate via fructose diphosphate aldolase and fructose diphosphatase whereas Neidhardt's P. aeruginosa ATCC 7700 could not.

The nature of the EMP defect in PA-1 was investigated in extracts of glucose- and lactate-grown cells. An extract of glucose-grown E. coli B was also prepared for comparison and as a check on the assay methods. Details of extract preparation and enzyme assay conditions are given in Materials and Methods. Table IX reveals that PA-1 was similar to Campbell's P. aeruginosa ATCC 9027. Both strains lacked phosphofructokinase but retained the necessary enzymes for conversion of triose phosphate to hexose phosphate.

Control of the synthesis of enzymes related to glucose metabolism in PA-1 was investigated by determining enzyme levels in extracts of cells grown on glucose, gluconate, glycerol, lactate and succinate minimal media. Enzyme profiles of the extracts are presented in Table X.

Glucose and gluconate degradation converge at the level of 6-phosphogluconate which is also the branch point of the ED and HMP pathways (cf. Figure 1). The glucose specific enzymes, glucokinase and

TABLE IX

ENZYMES OF THE EMP PATHWAY IN
PA-1 AND *E. COLI* B

	Specific Activity (n moles/min/mg protein)		
	PA-1		*E. coli* B
Enzyme	Glucose	Lactate	Glucose
Glucokinase	97.5	14.8	
Phosphohexose isomerase	57.8	49.3	
Phosphofructokinase	0.0	0.0	57.5
Fructose diphosphate aldolase	18.0	18.0	108.
Fructose diphosphatase	22.4	16.1	

Glucose and lactate were growth substrates for cells from which extracts were prepared.

TABLE X

ENZYMES RELATED TO GLUCOSE METABOLISM IN PA-1 GROWN ON VARIOUS SUBSTRATES

	Specific Activity (n moles/min/mg protein)				
	Growth Medium				
Enzyme	Glc	Gln	Gly	Lact	Suc
Glucose deHase	13.2	34.9	103.	4.9	6.3
Gluconate deHase	424.	104.	10.4	3.6	9.0
Glucokinase	97.5	67.7	82.2	14.8	8.5
Gluconokinase	44.0	37.4	0.0	2.7	0.0
Glucose 6-P deHase	276.	199.	194.	5.8	3.7
6-P-Gluconate deHase	89.5	31.3	18.2	0.0	0.0
Dehydrase	120.	61.3	80.1	1.5	2.6
KDPG Aldolase	304.*	$\geqslant$ 61.3	458.*	10.7*	8.5*
GAP deHase (NAD^+)	67.6	95.8	74.9	1.8	0.0
GAP deHase ($NADP^+$)	176.	184.	98.4	95.2	21.3
P-Glycerate Kinase	355.	289.	276.	363.	335.
Enolase	240.	208.	114.	232.	59.4
Pyruvate Kinase	168.	147.	101.	153.	151.
FDP Aldolase	16.0	13.4	17.2	15.2	16.9
FDPase	22.9	18.0	12.5	14.6	13.7
P-Hexose Isomerase	88.4	73.3	81.2	39.6	54.2

Abbreviations are as follows: Glc, glucose; Gln, gluconate; Gly, glycerol; Lact, lactate; Suc, succinate; deHase, dehydrogenase; P, phosphate or phospho-; KDPG, 2-keto-3-deoxy-6-phosphogluconate; GAP, glyceraldehyde 3-phosphate; FDP, fructose diphosphate; FDPase, fructose diphosphatase.

*KDPG aldolase was determined directly with KDPG.

glucose 6-phosphate dehydrogenase, were present at low basal levels in lactate and succinate grown cells. These activities were induced during growth on glucose, gluconate and glycerol. The gluconate specific enzyme, gluconokinase, was essentially absent in glycerol, lactate and succinate grown cells but was induced during growth on glucose and gluconate. The unique ED enzymes, 6-phosphogluconate dehydrase and KDPG aldolase, were present at low basal levels in lactate and succinate grown cells. Both enzymes were induced during growth on glucose, gluconate and glycerol. The enzyme catalyzing the first committed step of the HMP pathway, 6-phosphogluconate dehydrogenase, was not detectable in extracts of lactate and succinate grown cells but was induced during growth on glucose, gluconate and glycerol.

The enzymes of direct hexose oxidation, glucose dehydrogenase and gluconate dehydrogenase, have been regarded as a metabolic side issue off the main pathways of glucose and gluconate degradation (Sokatch, 1969). To further elucidate the physiological significance of these activities, glucose and gluconate dehydrogenases were also determined in extracts of cells grown on nutrient broth and nutrient broth containing 0.5 per cent glucose or gluconate and cells grown on lactate and induced in 0.5 per cent glucose for 4 hours. This additional information is summarized in Table XI.

Both glucose dehydrogenase and gluconate dehydrogenase were detected at low basal levels in extracts of nutrient broth, lactate and succinate grown cells (cf. Tables X and XI). Glucose dehydrogenase was only slightly increased by growth on glucose and induced to a relatively low level during growth on gluconate. Neither glucose nor gluconate induced glucose dehydrogenase in the presence of nutrient

TABLE XI

INDUCTION OF GLUCOSE AND GLUCONATE DEHYDROGENASES IN PA-1 AND 707

Strain	Growth Medium	Specific Activity (n moles/min/mg protein)	
		Glucose Dehydrogenase	Gluconate Dehydrogenase
PA-1	Glc NB	6.6	36.7
PA-1	Gln NB	1.8	131.
PA-1	NB	2.6	6.2
PA-1	Glc	13.0	245.
PA-1	Gln	24.8	71.4
PA-1	Lact	4.9*	2.4
PA-1	Lact/Glc	61.2	150.
707	Glc NB	77.3*	159.*

Abbreviations are as given in Table X and as follows: Glc NB, nutrient broth containing 0.5 per cent glucose; Lact/Glc, lactate grown and glucose induced.
*Value determined in a single extract; all other values are the average of two extracts.

broth. Paradoxically, growth on glycerol induced a high level of glucose dehydrogenase but failed to induce gluconate dehydrogenase. This exactly paralleled the response of the glucose and gluconate specific segments of the ED pathway to glycerol. Thus, neither glucose nor gluconate was either necessary or sufficient for induction of glucose dehydrogenase. In contrast, gluconate dehydrogenase was induced to a high level during growth on glucose and to a lower level during growth on gluconate. The presence of nutrient broth decreased induction by glucose but increased induction by gluconate.

Glucose dehydrogenase and gluconate dehydrogenase are apparently induced by different compounds since conditions were found which induced each activity exclusively. The fact that glucose dehydrogenase was induced by glycerol but not by direct exposure to glucose suggested that its synthesis was responsive to the intracellular level of some intermediate rather than to glucose itself.

The concept that glucose dehydrogenase is induced by an accumulated intermediate was supported by two additional observations. If PA-1 was grown on lactate and then induced in 0.5 per cent glucose for 4 hours, a high level of glucose dehydrogenase was attained (cf. Table XI). Accumulation of intermediates might be expected under these conditions since the glucose catabolic enzymes must be induced and the culture was probably somewhat oxygen limited due to its high cell density. Furthermore, in contrast to PA-1, strain 707 induced a high level of glucose dehydrogenase during growth on nutrient broth containing 0.5 per cent glucose. This strain was subsequently shown to lack 6-phosphogluconate dehydrase and would be expected to accumulate intermediates. Although these conditions also induced gluconate dehydrogenase, it is not clear

whether this was due to accumulation of a main pathway intermediate or of gluconate via glucose dehydrogenase.

Glycerol degradation and the ED pathway converge at the level of glyceraldehyde 3-phosphate (GAP) which is further degraded to pyruvate via the triose phosphate (TP) pathway (lower segment of the EMP pathway). Tiwari and Campbell (1969) have reported both NAD^+- and $NADP^+$-linked dehydrogenase activity in P. aeruginosa. The $NADP^+$-linked activity was greater than the NAD^+-linked activity and both were increased approximately 5-fold in glucose compared to succinate minimal medium. The nature of the dual nucleotide specificity was not investigated and its physiological significance was not indicated.

Both NAD^+- and $NADP^+$-linked GAP dehydrogenase activities were detected in PA-1. The $NADP^+$-linked activity was present at relatively high levels in extracts of glycerol-, lactate- and succinate-grown cells and was increased in extracts of glucose- and gluconate-grown cells. The NAD^+-linked activity was essentially absent in lactate- and succinate-grown cells but was induced during growth on glucose, gluconate and glycerol. These results indicated the existence of two different GAP dehydrogenases. The NAD^+-linked GAP dehydrogenase was induced during growth on substrates which are degraded at least in part via GAP and it thus appeared to be a typical catabolic enzyme. The $NADP^+$-linked GAP dehydrogenase was available for catabolism or anabolism depending on the growth substrate and was therefore amphibolic.

Phosphoglycerate kinase, enolase and pyruvate kinase were present at high levels under all conditions tested. Phosphoglycerate kinase and enolase were presumably amphibolic, functioning in both catabolism

and biosynthesis of triose phosphate.

The enzymes of the upper segment of the EMP pathway, fructose diphosphate aldolase and phosphohexose isomerase, in conjunction with fructose diphosphatase, apparently effect the conversion of triose phosphate to hexose phosphate. Fructose diphosphate aldolase and fructose diphosphatase were present at relatively low levels under all conditions tested. Phosphohexose isomerase activity was somewhat higher in extracts of lactate- and succinate-grown cells and was further increased during growth on glucose, gluconate and glycerol.

In summary, the main pathways from glucose and gluconate to 6-phosphogluconate, the unique ED enzymes, 6-phosphogluconate dehydrogenase and the NAD^+-linked GAP dehydrogenase were all induced by either glucose or gluconate. Interestingly, glycerol also induced all these activities except gluconokinase. This suggested that glycerol was metabolized at least in part via hexose phosphate. The reason for this was not clear but the fact that many glucose mutants were also glycerol-negative suggested that this may be an obligatory route for glycerol degradation. In addition, glycerol induced glucose dehydrogenase but not gluconate dehydrogenase. PA-1 also possessed the necessary enzymes for hexose phosphate biosynthesis from phosphoenolpyruvate by reversal of the EMP sequence.

The glucose 6-phosphate dehydrogenase of P. aeruginosa ATCC 7700 has been purified and characterized by Lessie and Neidhardt (1967a). The enzyme was nonspecific with respect to pyridine nucleotide coenzyme but preferred $NADP^+$. ATP and several other nucleoside triphosphates inhibited the enzyme by decreasing the apparent binding of substrate but no effect of ATP on pyridine nucleotide specificity could be shown.

Crude extracts of PA-1 also exhibited both NAD^+- and $NADP^+$-linked glucose 6-phosphate dehydrogenase activities as shown in Table XII. Pyridine nucleotide transhydrogenase (specific activity 46.4 n moles/min/mg protein) was not sufficient to account for the observed rates in the lactate-grown, glucose-induced PA-1 extract. Furthermore, if transhydrogenase were involved, a higher rate of NAD^+ reduction would have been expected in the lactate-grown PA-1 extract where transhydrogenase (specific activity 13.0 n moles/min/mg protein) would not have been rate limiting. NAD^+ was not contaminated with $NADP^+$ since the former was inactive with commercial yeast glucose 6-phosphate dehydrogenase which was specific for $NADP^+$. The ratio of NAD^+- to $NADP^+$-linked activity was approximately 0.7 in each extract assayed in Tris buffer, pH 8.0. Several mutants which failed to form glucose 6-phosphate dehydrogenase as measured by $NADP^+$ reduction showed no more than the expected rate of NAD^+ reduction. If the two activities were due to different enzymes, they were controlled in parallel. More likely, both activities were due to a single enzyme as discussed above. In this connection, it is interesting to note the effect of phosphate buffer on pyridine nucleotide specificity. When assayed in phosphate buffer, pH 7.5, the NAD^+-linked activity was virtually abolished while the $NADP^+$-linked activity was reduced to approximately 38 per cent of that observed in Tris buffer at the same pH. This phenomenon was not further investigated but may be of interest as a possible control mechanism in conjunction with ATP for determining both activity and pyridine nucleotide specificity of glucose 6-phosphate dehydrogenase in P. aeruginosa.

TABLE XII

PYRIDINE NUCLEOTIDE SPECIFICITY OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE

Strain	Growth Medium	Assay		Specific Activity (n moles/min/mg protein)	
		Buffer	pH	NAD^+	$NADP^+$
PA-1	Lactate	Tris	8.0	3.6	5.8
PA-1	Glucose	Tris	8.0	170.0	233.
707	Lact/Glc	Tris	8.0	30.6	47.9
PA-1	Lact/Glc	Tris	8.0	133.	179.
PA-1	Lact/Glc	Tris	7.5	116.	138.
PA-1	Lact/Glc	Phosphate	7.5	0.5	51.9

Lact/Glc indicates that cells were grown on lactate and induced by exposure to 0.5 per cent glucose for 4 hours.

Mutant strains selected for enzyme analysis were chosen on the basis of transductional groupings. Since certain strains lysed during growth on nutrient broth in the presence of glucose, a standard procedure for extract preparation was adopted where lactate-grown cells were induced by exposure to the desired substrate for 4 hours as detailed in Materials and Methods. Glucose, gluconate and glycerol were used as inducers. Extracts of glycerol-grown cells were also prepared for those strains which retained the ability to grow on glycerol.

Enzymes of the upper segment of the EMP pathway and the TP pathway were determined only in extracts of glucose-induced cells. These results are presented in Table XIII. The exceptionally low levels of $NADP^+$-linked GAP dehydrogenase in 707, 730 and 736 were due to enzyme degradation during the induction period since these cultures exhibited specific activities of 95.0, 58.5 and 77.7 n moles per min per mg protein respectively after growth on lactate prior to induction with glucose. All the mutants retained the enzymes necessary for conversion of phosphoenolpyruvate to hexose phosphate consistent with their wild type growth on lactate and other glucogenic substrates. Mutants 707, 718 and 732 were able to grow on glycerol; all other mutants were glycerol-negative glucose mutants. The possibility that failure to grow on both glucose and glycerol was due to pyruvate kinase deficiency was eliminated since none of the mutants lacked this activity.

The inducible enzymes involved in direct hexose oxidation, catabolism of glucose and gluconate via the ED pathway, 6-phosphogluconate dehydrogenase, and NAD^+-linked GAP dehydrogenase were determined in extracts of cells grown on lactate and induced with glucose, gluconate or glycerol. Extracts of glycerol-grown 707, 718 and 732 were also

TABLE XIII

TP AND EMP PATHWAY ENZYMES IN EXTRACTS OF GLUCOSE INDUCED MUTANTS

Strain	Specific Activity (n moles/min/mg protein)						
	GPD ($NADP^+$)	PGK	ENL	PYK	FDA	FDP	PHI
PA-1	155.	306.	173.	123.	18.1	26.6	52.4
707	38.6	387.	99.5	126.	19.3	19.6	45.6
718	62.2	260.	203.	50.2	16.4	12.9	67.1
732	114.	399.	147.	63.9	13.1	12.9	72.8
760	159.	480.	279.	126.	20.4	22.3	66.4
773	163.	467.	148.	122.	15.7	8.3	71.7
777	166.	492.	118.	127.	15.1	10.7	74.1
734	105.	372.	74.0	116.	11.3	7.2	66.8
738	52.5	398.	166.	120.	16.4	17.8	69.4
730	20.0	212.	132.	118.	14.5	15.0	68.3
736	9.1	353.	138.	126.	13.0	19.6	67.7
720	98.7	460.	172.	113.	13.8	18.9	83.2
755	74.7	426.	185.	123.	15.5	20.2	106.

Abbreviations are as follows: GPD ($NADP^+$), $NADP^+$-linked glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; ENL, enolase; PYK, pyruvate kinase; FDA, fructose diphosphate aldolase; FDP, fructose diphosphatase; PHI, phosphohexose isomerase.

examined. These results are presented in Tables XIV through XVIII. Each table presents strains with similar enzyme profiles.

Profiles of strains 707, 718 and 732 (707 group) are given in Table XIV. These strains grew on glycerol although 732 was quite slow (cf. Table III). Each strain lacked 6-phosphogluconate dehydrase. This was established in the following manner. Each strain was inactive in the double sensitivity coupled dehydrase-aldolase assay. The presence of KDPG aldolase in each strain was established either by direct assay with KDPG or by coupling with an extract of 720 as described in Materials and Methods. Furthermore, addition of excess KDPG aldolase in the form of an extract of E. coli B grown on nutrient broth containing 0.5 per cent glucose did not restore activity in the double sensitivity, coupled dehydrase-aldolase assay. The E. coli B extract had KDPG aldolase (specific activity 119 n moles per min per mg protein) as determined by direct assay with KDPG but undetectable 6-phosphogluconate dehydrase as determined by the double sensitivity, coupled dehydrase-aldolase assay. Since these techniques were able to detect basal levels of dehydrase and KDPG aldolase, it was concluded that the strains lacked 6-phosphogluconate dehydrase. Strain 718, which exhibited low 6-phosphogluconate dehydrase activity detected with its own KDPG aldolase in the extract of glycerol-grown cells, was known to be leaky.

6-Phosphogluconate dehydrogenase was present only in extracts of glycerol-grown cells. The 4-hour exposure to glycerol was not sufficient for induction. Interestingly, the ability to grow on glycerol appeared to be related to 6-phosphogluconate dehydrogenase. Strains 707 and 718 which grew normally on glycerol had higher levels of this

TABLE XIV

INDUCIBLE ENZYMES OF GLUCOSE METABOLISM IN 707 AND RELATED STRAINS

	Specific Activity (n moles/min/mg protein)											
	707				718				732			
Enzyme	Glc	Gln	Gly	Gly	Glc	Gln	Gly	Gly	Glc	Gln	Gly	Gly
Glucose deHase	45.8	18.4	26.6	43.6	21.3	22.2	33.3	66.9	26.4	17.0	39.2	146.
Gluconate deHase	17.8	10.8	40.7	26.3	20.2	15.9	41.3	36.0	22.5	15.7	44.6	2.1
Glucokinase	25.1	28.0	36.1	91.2	30.0	2.6	28.9	80.1	36.4	5.1	25.2	18.9
Gluconokinase	8.6	7.5	24.2	74.7	6.7	0.0	11.4	10.8	9.8	2.1	8.6	4.0
Glc 6-P deHase	46.4	40.4	65.4	363.	48.7	36.0	66.4	311.	61.7	38.6	52.7	145.
6-P Gln deHase	0.0	0.0	0.0	7.6	0.0	0.0	0.0	5.4	0.0	0.0	0.0	0.6
Dehydrase	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.0	0.0	0.0	0.0
KDPG Aldolase	105.*	8.7	13.6	630.*	10.7	15.6	20.2	$\geqslant$7.1	11.8	10.4	15.9	42.3
GAP deHase (NAD^+)	3.5	8.9	20.4	166.	6.4	5.3	17.6	80.9	24.0	11.9	18.9	147.

Each strain was grown on lactate and induced with the indicated substrate; extracts of cells grown on glycerol are designated Gly. Abbreviations are as given in Table X. KDPG aldolase was determined directly with KDPG (*) or by coupling with added dehydrase as described in the text.

activity than strain 732 which grew slowly and exhibited a barely detectable level of 6-phosphogluconate dehydrogenase. Correlated with the appearance of 6-phosphogluconate dehydrogenase during growth on glycerol was an increase of glucose 6-phosphate dehydrogenase, NAD^+-linked GAP dehydrogenase and KDPG aldolase.

Several quantitative differences were apparent among the strains of the 707 group. In 707, exposure to gluconate induced higher levels of glucokinase and gluconokinase than in 718 or 732. Growth on glycerol induced higher levels of glucokinase in 707 and 718 than in 732. In addition, glycerol induced the gluconate specific enzymes gluconate dehydrogenase and gluconokinase to various extents in each strain. Gluconokinase was higher in extracts of glycerol-grown 707 than in extracts of glycerol grown 718 and 732. In each strain, gluconate dehydrogenase activity was greater than glucose dehydrogenase after 4 hours exposure to glycerol. However, in extracts of glycerol-grown cells, this was reversed and in 732 returned to wild type levels. The basal levels of NAD^+-linked GAP dehydrogenase in extracts of lactate-grown 707, 718 and 732 were 3.2, 3.5 and 4.2 n moles per minute per mg protein, respectively. This activity was induced by glycerol in 707 and 718 and by glucose and glycerol in 732. Thus each strain of the 707 group exhibited some unique responses to the conditions of growth and induction employed.

Finally it should be noted that neither 6-phosphogluconate dehydrase nor KDPG aldolase was necessary for growth on glycerol.

Table XV presents the enzyme profiles of strains 760, 773, 777, and 728 (760 group). Strain 728 was only partially characterized. These strains lacked 6-phosphogluconate dehydrase and 6-phosphogluconate

TABLE XV

INDUCIBLE ENZYMES OF GLUCOSE METABOLISM IN 760 AND RELATED STRAINS

	Specific Activity (n moles/min/mg protein)									
	760			773			777			728
Enzyme	Glc	Gln	Gly	Glc	Gln	Gly	Glc	Gln	Gly	Glc
Glucose deHase	22.6	13.2	48.5	9.7	8.2	45.2	10.8	10.4	44.7	18.8
Gluconate deHase	7.2	10.2	32.4	6.2	6.8	28.4	5.6	7.8	20.0	8.4
Glucokinase	40.7	35.0	34.2	38.6	33.7	30.2	7.1	8.8	7.6	34.4
Gluconokinase	64.0	64.6	63.3	65.1	59.2	58.7	70.6	72.9	68.4	68.5
Glc 6-P deHase	44.2	39.9	42.9	41.9	31.7	30.8	48.6	42.9	37.5	50.0
G-P Gln deHase	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dehydrase	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KDPG Aldolase	15.9	10.2	10.2	13.5	10.1	11.3	14.2	12.9	10.9	13.3
GAP deHase (NAD^+)	20.4	10.2	6.9	16.0	8.5	5.3	13.6	9.8	6.1	---

Each strain was grown on lactate and induced with the indicated substrate. Abbreviations are as given in Table X. KDPG aldolase was determined by coupling with added dehydrase as described in the text.

dehydrogenase. Unlike the 707 group, these strains failed to grow on glycerol. Since 6-phosphogluconate dehydrase was not required for growth on glycerol but formation of 6-phosphogluconate dehydrogenase appeared to be related to growth on glycerol, the failure of these strains to grow on glycerol was attributable to lack of 6-phosphogluconate dehydrogenase. These strains produced high levels of gluconokinase under all conditions of induction (including glycerol). A slight induction of gluconate dehydrogenase by glycerol was also noted. Strain 777 was unique in that it exhibited only the basal level of glucokinase under all conditions of induction.

Although glucose appeared to induce NAD^+-linked GAP dehydrogenase in these strains, it actually did not. Extracts of 760, 773 and 777 grown on lactate had specific activities of 16.5, 14.2 and 12.5 n moles per minute per mg protein, respectively, for this enzyme. Thus, strains of the 760 group were either able to induce NAD^+-linked GAP dehydrogenase endogenously or were low level constitutive mutants.

Enzyme profiles of 734 and 738 (734 group) are presented in Table XVI. These strains lacked 6-phosphogluconate dehydrase, 6-phosphogluconate dehydrogenase and gluconokinase. Strains 734 and 738 differ from each other in several respects. The glycerol catabolic enzyme L-α-glycerol phosphate dehydrogenase could not be detected in the extract of glycerol-induced 734 (K. Brown, personal communication). Thus, glycerol was a poor inducer in 734 as reflected by the low levels of glucose dehydrogenase and gluconate dehydrogenase compared to those of 738. Furthermore, glycerol induction of 734 produced lower levels of glucokinase and glucose 6-phosphate dehydrogenase than did induction with glucose. Strain 734 also responded poorly to gluconate induction

TABLE XVI

INDUCIBLE ENZYMES OF GLUCOSE METABOLISM IN 734 AND 738

	Specific Activity (n moles/min/mg protein)					
	734			738		
Enzyme	Glc	Gln	Gly	Glc	Gln	Gly
Glucose deHase	22.5	22.5	14.4	26.9	20.5	49.6
Gluconate deHase	20.7	9.1	9.3	11.4	11.1	132.
Glucokinase	30.9	0.0	6.9	13.7	9.7	10.8
Gluconokinase	0.0	0.0	0.0	0.0	0.4	0.0
Glc 6-P deHase	33.6	21.4	15.5	8.7	4.4	5.1
6-P Gln deHase	0.0	0.0	0.0	0.0	0.0	0.3
Dehydrase	0.0	0.0	0.0	0.0	0.0	0.0
KDPG Aldolase	10.5	8.7	7.5	5.0	1.7	3.1
GAP deHase (NAD^+)	11.0	6.4	4.6	0.0	6.9	5.4

Each strain was grown on lactate and induced with the indicated substrate. Abbreviations are as given in Table X. KDPG aldolase was determined by coupling with added dehydrase as described in the text.

which was probably due to its limited ability to metabolize gluconate. In strain 738, glucose dehydrogenase was induced by glycerol and to a lesser extent by glucose and gluconate. Glycerol also induced a high level of gluconate dehydrogenase in this strain. However, the remaining main pathway enzymes, glucokinase and glucose 6-phosphate dehydrogenase, were present at only basal levels under all conditions of induction.

Strains 730 and 736 exhibited the most extensive damage of all the glucose mutants. These strains were suspected deletion mutants. Spontaneous glucose revertants were never observed for either strain and NTG mutagenesis of 736 failed to produce glucose revertants. As shown in Table XVII, both strains lacked 6-phosphogluconate dehydrase, 6-phosphogluconate dehydrogenase, gluconokinase, glucose dehydrogenase and gluconate dehydrogenase. As noted previously, $NADP^+$-linked GAP dehydrogenase was degraded during induction (cf. Table XVIII). The absence of glucose and gluconate dehydrogenases in these extracts was not due to degradation of basal levels during induction since these activities were undetectable in an extract of lactate-grown 730 and an extract of 736 grown on nutrient broth containing 0.5 per cent glucose. Basal levels of glucose and gluconate dehydrogenases were detectable in extracts of PA-1 grown on lactate or nutrient broth (cf. Table XI). Several differences between 730 and 736 have been noted. Strain 730 was apparently permeable to glucose whereas 736 was not. Extracts of glycerol-induced cells revealed low levels of glycerol kinase and L - α-glycerol phosphate dehydrogenase in 730 but the latter activity was undetectable in 736 (K. Brown, personal communication). Thus, the low levels of glucokinase and glucose 6-phosphate dehydrogenase

TABLE XVII

INDUCIBLE ENZYMES OF GLUCOSE METABOLISM IN 730 AND 736

	Specific Activity (n moles/min/mg protein)					
	730			736		
Enzyme	Glc	Gln	Gly	Glc	Gln	Gly
Glucose deHase	0.0	0.3	0.0	0.5	0.0	0.0
Gluconate deHase	0.6	0.6	0.0	0.0	1.1	0.3
Glucokinase	18.0	17.7	19.8	10.2	10.4	9.0
Gluconokinase	0.0	0.0	0.0	0.0	0.0	0.0
Glc 6-P deHase	8.5	14.3	13.9	1.7	6.9	2.1
6-P Gln deHase	1.0	0.0	0.0	0.0	0.0	0.5
Dehydrase	0.0	0.0	0.0	0.0	0.0	0.0
KDPG Aldolase	5.5	3.0	5.2	2.5	3.0	6.6
GAP deHase (NAD^+)	1.5	3.4	5.0	1.1	0.0	1.1

Each strain was grown on lactate and induced with the indicated substrate. Abbrevations are as given in Table X. KDPG aldolase was determined by coupling with added dehydrase as described in the text.

observed in each 736 extract probably represent partially degraded basal levels of each enzyme. Although 730 was permeable to glucose and could metabolize glycerol at least to a limited extent, only slightly elevated levels of these activities were detected in the 730 extracts.

The enzyme profiles of strains 720 and 755 (720 group) are given in Table XVIII. Mutants of this group lacked KDPG aldolase rather than 6-phosphogluconate dehydrase. This was demonstrated by direct assay with KDPG in extracts of glucose-induced cells for both strains. The absence of KDPG aldolase in extracts of gluconate- or glycerol-induced cells of both strains was demonstrated by restoration of activity to the double sensitivity, coupled dehydrase-aldolase assay upon addition of KDPG aldolase in the form of an extract of E. coli B grown on nutrient broth containing 0.5 per cent glucose. Both strains had barely detectable gluconokinase activity following induction with each substrate. Trace activity of 6-phosphogluconate dehydrogenase was detected in each strain only after induction with glycerol.

Glucose 6-phosphate dehydrogenase activity was quite low in 720 compared to 755 under all conditions of induction. Furthermore, this activity was undetectable in an extract of lactate-grown 720 where PA-1 showed a detectable basal level. The high levels of NAD^+-linked GAP dehydrogenase found in extracts of glucose-induced 755 and especially 720 were interesting but somewhat misleading. Extracts of lactate grown 720 and 755 exhibited specific activities of 47.9 and 37.9 n moles per minute per mg protein for this enzyme. This was in sharp contrast to PA-1 where an extract of lactate-grown cells had only a low basal level of this enzyme (cf. Table X). Thus, NAD^+-linked GAP dehydrogenase was endogenously induced or became constitutive in

TABLE XVIII

INDUCIBLE ENZYMES OF GLUCOSE METABOLISM IN 720 AND 755

	Specific Activity (n moles/min/mg protein)					
	720			755		
Enzyme	Glc	Gln	Gly	Glc	Gln	Gly
Glucose deHase	23.0	21.0	35.1	23.5	31.7	50.4
Gluconate deHase	25.4	29.4	90.6	14.2	16.1	53.3
Glucokinase	90.9	63.9	60.8	93.5	56.4	56.2
Gluconokinase	0.5	1.5	0.5	0.7	0.6	0.3
Glc 6-P deHase	2.9	6.0	8.6	181.	138.	158.
6-P Gln deHase	0.0	0.0	1.0	0.0	0.0	0.3
Dehydrase	53.2	39.3	28.0	38.0	18.8	20.5
KDPG Aldolase	0.0*	0.0	0.0	0.0*	0.0	0.0
GAP deHase (NAD^+)	109.	17.5	16.3	39.0	6.5	6.1

Each strain was grown on lactate and induced with the indicated substrate. Abbreviations are as given in Table X. KDPG aldolase was assayed directly with KDPG (*) or by coupling with added dehydrase as described in the text.

mutants 720 and 755. Therefore, exposure to glucose actually caused a two-fold increase of this activity in 720 and merely maintained the basal level in 755. Furthermore, it became clear that exposure to gluconate and glycerol failed to induce NAD^+-linked GAP dehydrogenase and that the observed levels were actually degraded basal levels.

Another significant property of the 720 group was lysis during growth on nutrient broth containing 0.5 per cent glucose (cf. Table V). This was presumably due to accumulation of KDPG rather than glucose 6-phosphate since both 720 and 755 exhibited the phenomenon. Furthermore, other strains (e.g., 734 and 738) with similar enzyme profiles but lacking 6-phosphogluconate dehydrase rather than KDPG aldolase did not lyse in glucose-supplemented nutrient broth.

Catabolite Repression of Histidase

Lessie and Neidhardt (1967b) showed that several enzymes of histidine catabolism were inducible and subject to catabolite repression in P. aeruginosa ATCC 7700. Histidase, the first enzyme of the sequence, has been useful in kinetic studies of induced enzyme synthesis in other systems where simple, sensitive whole cell assays were available (Hartwell and Magasanik, 1963; Schlesinger and Magasanik, 1965). Histidase could be assayed quite simply in whole cells of PA-1 which had been frozen and thawed, as described in Materials and Methods. This facilitated the investigation of catabolite repression of histidase in PA-1 and selected glucose mutants.

Catabolite repression of histidase by glucose or gluconate was demonstrated in PA-1 as follows. Nutrient broth-grown cells were washed and resuspended in 25 ml of 0.25 per cent histidine minimal

medium in each of three 250 ml side arm flasks. The cells were incubated at 37 C in a water bath shaker. When growth reached an OD of approximately 0.1, glucose and gluconate were added to a final concentration of 0.25 per cent to the second and third flasks, respectively. Samples of each culture (2 ml) and OD readings were taken at the time of addition and periodically thereafter. The culture samples were transferred immediately to precooled centrifuge tubes and the cells were harvested by centrifugation at 12,100 x g for five minutes at 0 C. The pellets were frozen and stored at -20 C until assayed.

Figure 10a shows the growth curves obtained in this experiment. Addition of glucose or gluconate stimulated growth only slightly. Figure 10b shows the amount of histidase per ml of culture as a function of culture OD. The slope of such a plot represents the differential rate of histidase synthesis, that is, the rate of histidase synthesis relative to the rate of total protein synthesis. A unit of enzyme was defined as that amount catalyzing the formation of 1 n mole of urocanate per minute under the specified assay conditions. PA-1 synthesized histidase at a differential rate of 138 units per ml per unit culture OD during growth on 0.25 per cent histidine. Addition of glucose and gluconate to 0.25 per cent repressed the differential rate of histidase synthesis to 40 and 44 units per ml per unit culture OD, respectively. This corresponded to 71 per cent repression by glucose and 68 per cent repression by gluconate. In similar experiments, 1 mM cyclic AMP did not relieve glucose or gluconate repression of histidase in PA-1.

Table XIX shows that histidase was undetectable in wild type cells grown on a variety of minimal media but was induced during growth on

Figure 10. Catabolite Repression of Histidase by Glucose and Gluconate in PA-1.

Nutrient broth-grown cells were washed and resuspended in 25 ml of 0.25 per cent histidine minimal medium in each of three 250 ml sidearm flasks. At the time indicated by the arrow, glucose and gluconate were added to 0.25 per cent to the second and third flasks respectively. Samples (2 ml) of each culture were taken at the time of addition and periodically thereafter and immediately transferred to precooled centrifuge tubes. The cells were collected by centrifugation at 0 C and the pellets were frozen at -20 C until assayed.

(a) Growth on histidine (o); growth on histidine after addition of glucose (△) or gluconate (□).

(b) Differential rate of histidase synthesis during growth on histidine (o) and after addition of glucose (△) or gluconate (□).

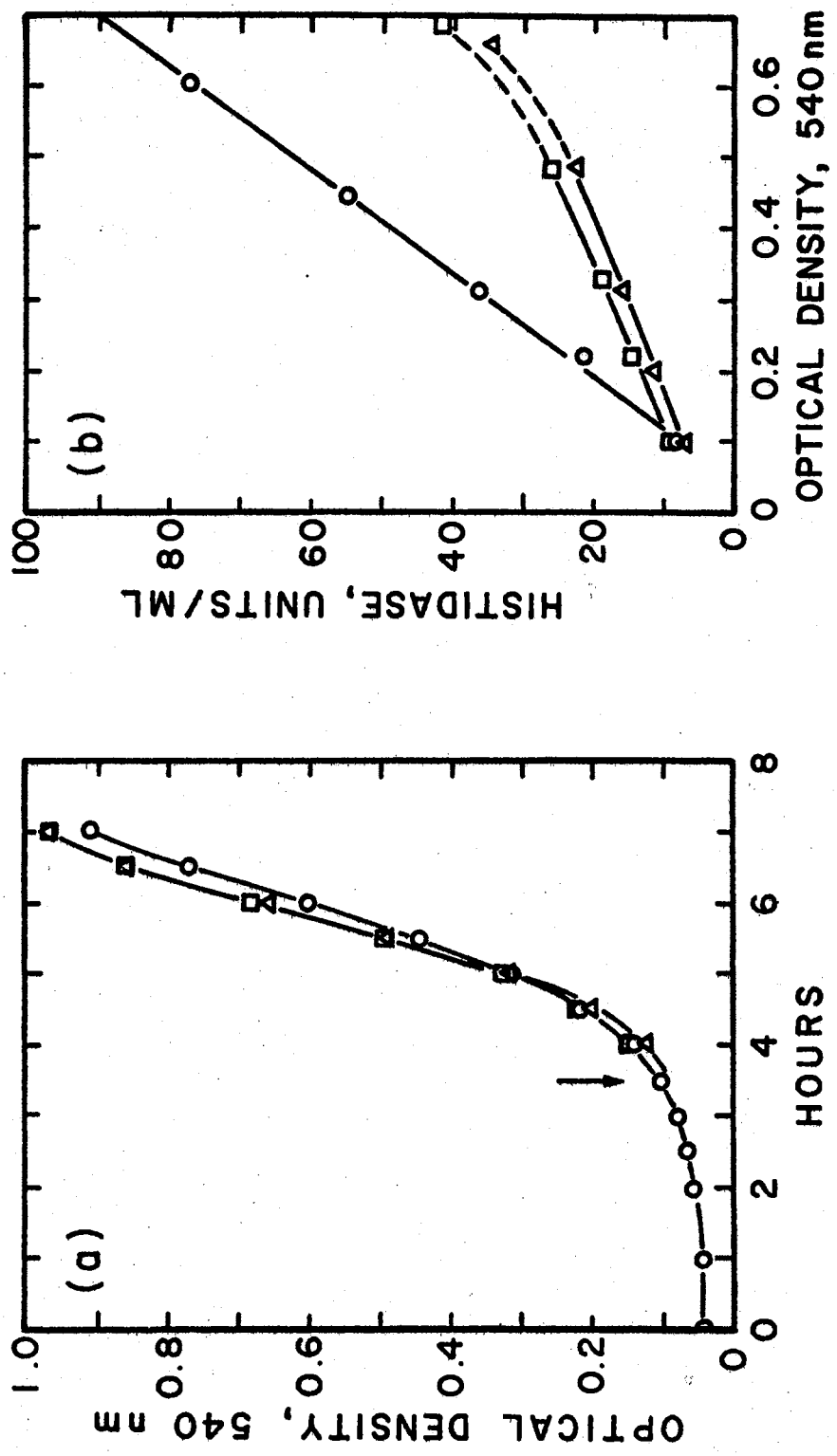

(b)
HISTIDASE, UNITS/ML
OPTICAL DENSITY, 540nm
0
20
40
60
80
100
0
0.2
0.4
0.6
(a)
OPTICAL DENSITY, 540 nm
HOURS
0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8

TABLE XIX

INDUCTION AND CATABOLITE REPRESSION
OF HISTIDASE IN PA-1

Growth Medium	Specific Activity (n moles/min-ml/unit culture OD) Histidase
Glucose	0.0
Gluconate	0.0
Glycerol	0.0
Lactate	0.0
Succinate	0.0
Histidine	115.
Urocanate	99.6
Histidine + Glucose	27.9
Urocanate + Glucose	24.7
Histidine + Gluconate	38.6
Histidine + Glycerol	81.9
Histidine + Lactate	63.8
Histidine + Succinate	16.8

All carbon sources were used at a concentration of 0.25 per cent.

either histidine or uroconate, the first intermediate of histidine degradation. Histidase was also repressed by other substrates to various extents. Succinate caused the most severe repression, followed by glucose and gluconate. Glucose repressed induction of histidase by histidine and urocanate to the same extent. Lactate and glycerol were weakly repressive.

The identity of the effector of glucose repression of histidase was investigated by monitoring histidase levels following addition of glucose or gluconate to glucose mutants growing on histidine. Strain 707, which lacked 6-phosphogluconate dehydrase and produced only low levels of 6-phosphogluconate dehydrogenase during growth on glycerol would be expected to accumulate 6-phosphogluconate and the preceding intermediates when exposed to either glucose or gluconate. Strain 730 retained the basal level of glucokinase and glucose 6-phosphate dehydrogenase but lacked glucose dehydrogenase, 6-phosphogluconate dehydrase, and 6-phosphogluconate dehydrogenase. Thus exposure to glucose should allow a slow accumulation of glucose 6-phosphate and 6-phosphogluconate. In addition, strain 730 lacked gluconate dehydrogenase and gluconokinase; gluconate would presumably enter the cells but would not be metabolized and no intermediates would accumulate. Finally, strain 736, which had the same enzyme profile as 730 but was in addition unable to transport glucose, should not accumulate intermediates from either glucose or gluconate.

Preliminary tests showed that control of histidase synthesis was not altered in these three strains. These data are given in Table XX. Histidase induction by histidine and repression by lactate and succinate were comparable to that observed in the wild type.

TABLE XX

INDUCTION AND CATABOLITE REPRESSION OF HISTIDASE IN 707, 730, AND 736

Growth Medium	Specific Activity (n moles/min-ml/unit culture OD) Histidase 707	730	736
Lactate	0.0	0.0	0.0
Succinate	0.0	0.0	0.0
Histidine	101.	112.	122.
Histidine + Lactate	56.4	56.9	55.3
Histidine + Succinate	10.6	8.5	10.0

All carbon sources were 0.25 per cent.

The differential rate of synthesis of histidase in 0.25 per cent histidine minimal medium following addition of glucose or gluconate to 0.25 per cent final concentration was determined for each mutant as described above for PA-1.

Figure 11a shows that addition of glucose or gluconate to 707 growing on histidine resulted in linear growth as noted previously. The differential rate of histidase synthesis in each culture is shown in Figure 11b. Addition of glucose and gluconate repressed the initial differential rate of histidase synthesis from 153 units per ml per unit culture OD for the histidine control to 57.5 and 52.5 units per ml per unit culture OD respectively. These results were comparable to those observed in the wild type and indicated that the effector was formed efficiently from either glucose or gluconate by 707. The abrupt cessation of histidase synthesis seen in the glucose repressed culture may have been due to accumulation of excess effector.

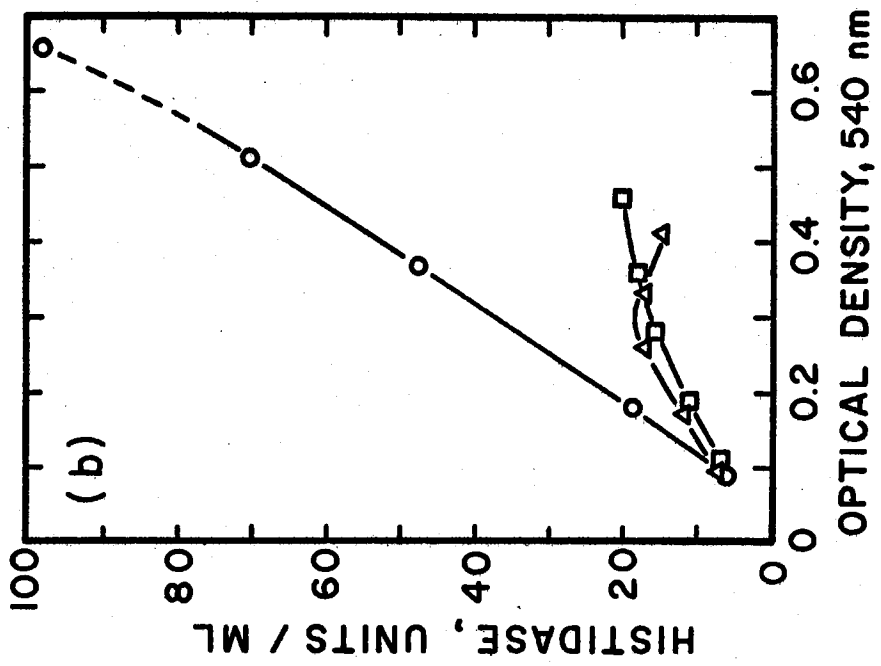
Growth curves and differential plots for 730 are presented in Figures 12a and 12b. The addition of glucose caused a slight inhibition of growth on histidine whereas gluconate was without effect. Glucose mildly repressed the differential rate of synthesis of histidase from 131 units per ml per unit culture OD for the control to 95.5 units per ml per unit culture OD. Thus the basal enzyme levels of 730 were able to generate the effector inefficiently from glucose. The differential rate of histidase synthesis following addition of gluconate was 144 units per ml per unit culture OD. This was slightly higher than the control differential rate but intermediate between the control differential rates observed for PA-1 and 707. Thus gluconate was unable to repress histidase in 730.

Figure 11. Catabolite Repression of Histidase by Glucose and Gluconate in 707.

The experimental procedure was the same as described in Figure 10.

(a) Growth on histidine (o); growth on histidine after addition of glucose (△) or gluconate (□).

(b) Differential rate of histidase synthesis during growth on histidine (o) and after addition of glucose (△) or gluconate (□).


(b)
HISTIDASE, UNITS / ML
OPTICAL DENSITY, 540 nm
0
20
40
60
80
100
0
0.2
0.4
0.6

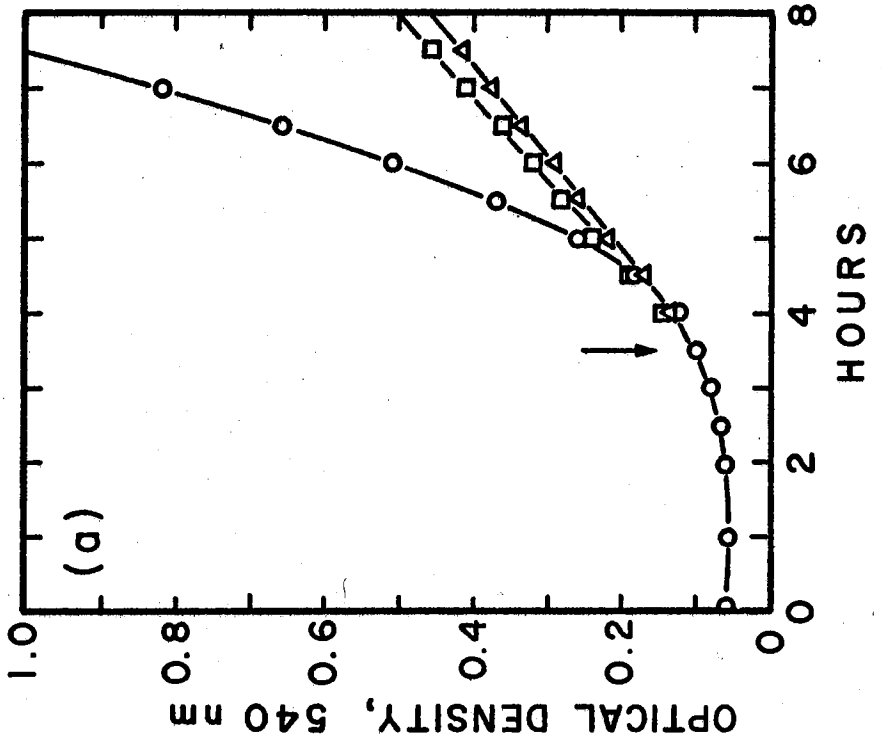
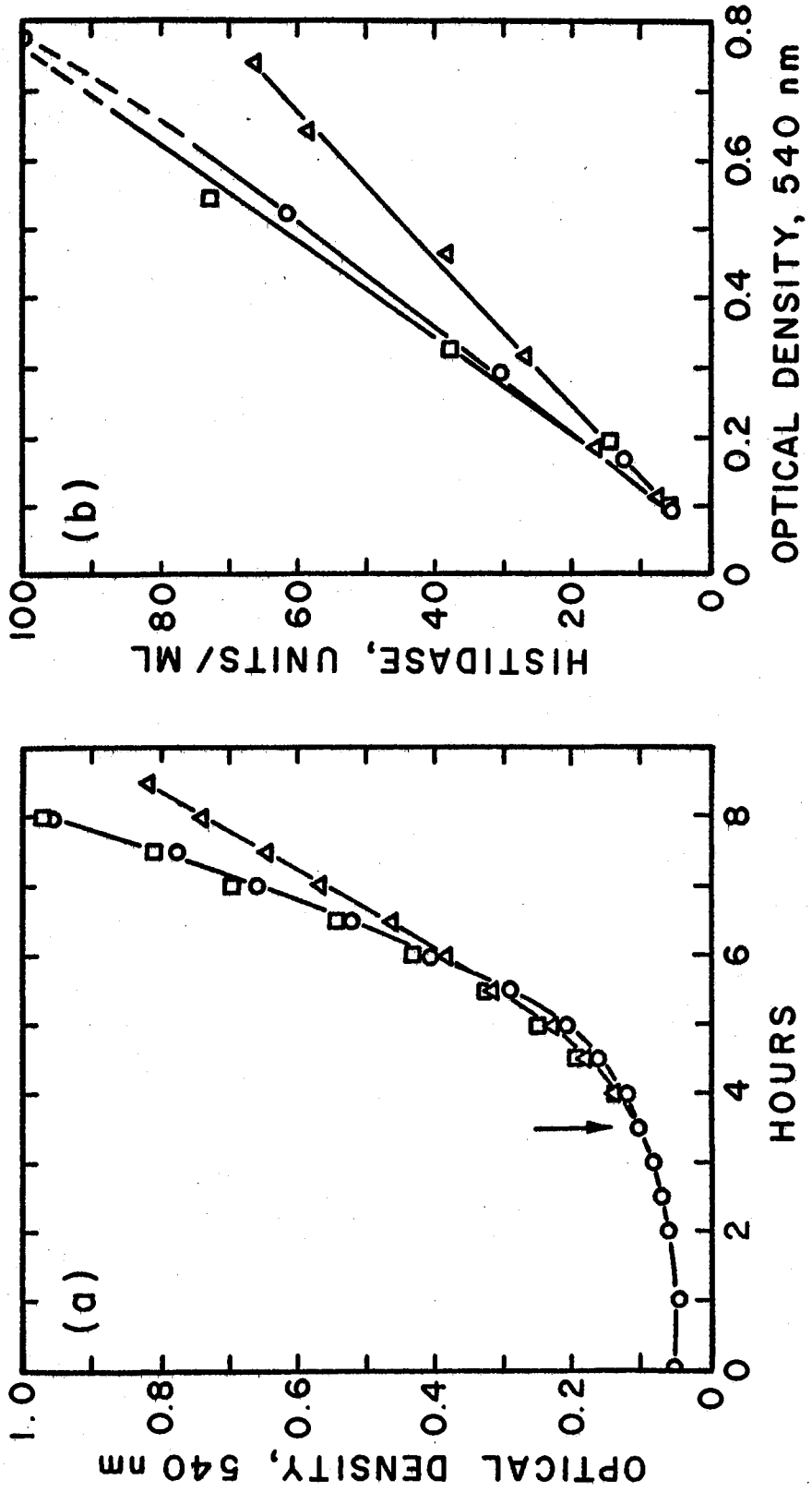

(a)
OPTICAL DENSITY, 540 nm
HOURS
0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8

Figure 12. Catabolite Repression of Histidase by Glucose and Gluconate in 730.

The experimental procedure was the same as described in Figure 10.

(a) Growth on histidine (o); growth on histidine after addition of glucose (△) or gluconate (□).

(b) Differential rate of histidase synthesis during growth on histidine (o) and after addition of glucose (△) or gluconate (□).


(a)
OPTICAL DENSITY, 540 nm
HOURS
(b)
HISTIDASE, UNITS/ML
OPTICAL DENSITY, 540 nm

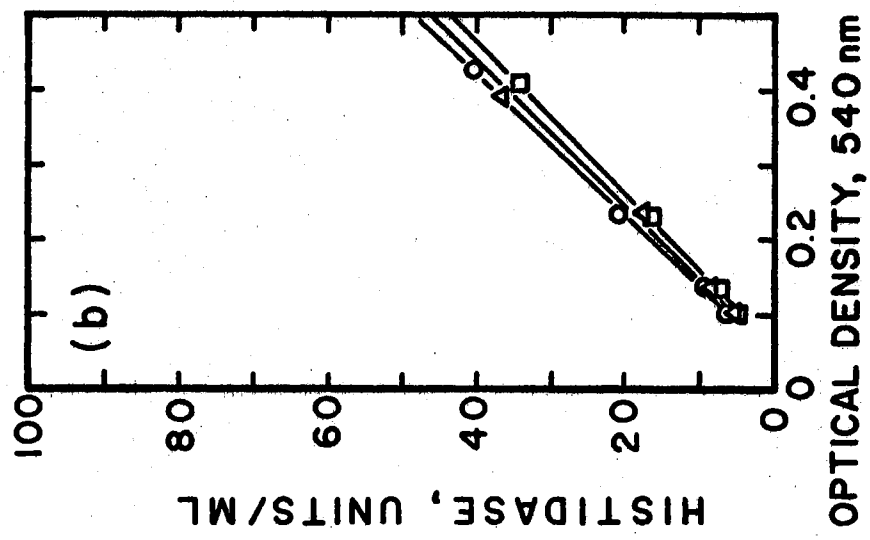
Addition of glucose or gluconate to 736 growing on histidine had essentially no effect on growth or differential rate of histidase synthesis as shown in Figures 13a and 13b. The differential rates of histidase synthesis in the histidine control and following addition of glucose and gluconate were 106, 104, and 98.5 units per ml per unit culture OD respectively. Thus 736, which had the same enzyme profile as 730, was unable to generate the effector even inefficiently from glucose due to its failure to enter the cells.

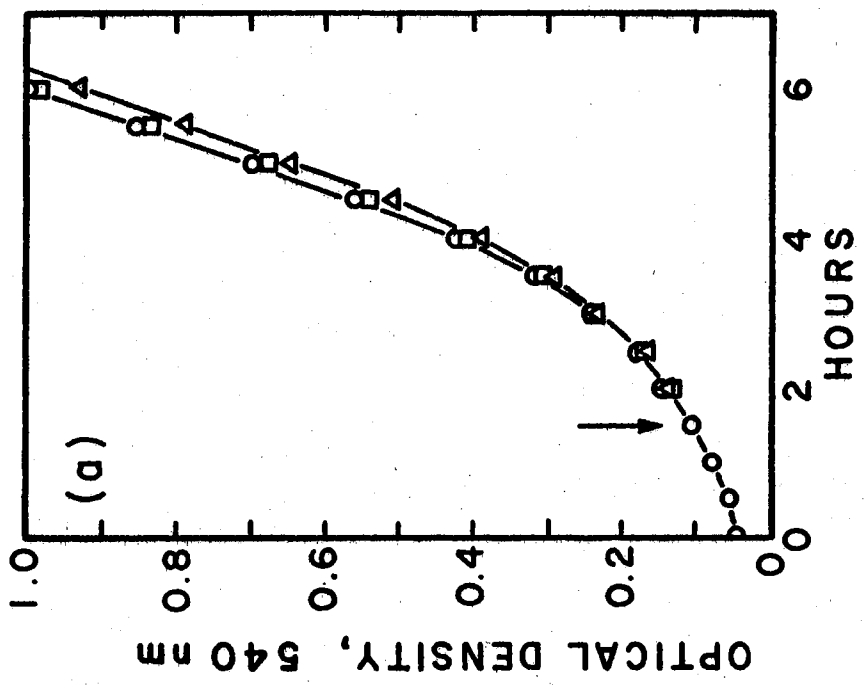
Figure 13. Catabolite Repression of Histidase by Glucose and Gluconate in 736.

The experimental procedure was the same as described in Figure 10.

(a) Growth on histidine (o); growth on histidine after addition of glucose (△) or gluconate (□).

(b) Differential rate of histidase synthesis during growth on histidine (o) and after addition of glucose (△) or gluconate (□).


(b)
HISTIDASE, UNITS/ML
OPTICAL DENSITY, 540 nm
0
20
40
60
80
100
0
0.2
0.4


(a)
OPTICAL DENSITY, 540 nm
HOURS
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6

CHAPTER IV

DISCUSSION

Regulation of the Synthesis of Enzymes of Glucose Metabolism

The pathways available for glucose catabolism in PA-1 were investigated by enzyme analysis in extracts of glucose-grown and lactate-grown cells. A functional EMP pathway did not exist in this organism since one of the two key enzymes, phosphofructokinase, was absent (Table IX). Tiwari and Campbell (1969) have shown that phosphofructokinase deficiency was the only defect associated with lack of a functional EMP pathway in P. aeruginosa ATCC 9027. Wood and Schwerdt (1954) excluded participation of the EMP pathway in glucose degradation by P. putida A.3.12 (ATCC 12633, formerly P. fluorescens) for the same reason. However, Lessie and Neidhardt (1967a) reported that P. aeruginosa ATCC 7700 lacked fructose diphosphate aldolase; no other EMP pathway enzymes were mentioned. Although this also eliminated the EMP pathway for glucose degradation, its implications for hexose and pentose biosynthesis were quite different. Extracts of glucose-grown PA-1 contained the appropriate activities for glucose catabolism via the ED and HMP pathways (Table X). This agreed with the results of previous studies (Wang et al., 1959; von Tigerstrom and Campbell, 1966; Lessie and Neidhardt, 1967a; Ng and Dawes, 1967; Tiwari and Campbell, 1969).

Growth requires a constant supply of hexose phosphate and pentose phosphate for cellular syntheses. During growth on glucose, hexose phosphate was available directly and pentose phosphate was formed oxidatively via 6-phosphogluconate dehydrogenase (Table X). Cells growing on gluconate also formed pentose phosphate oxidatively; hexose phosphate was presumably formed from pentose phosphate by the nonoxidative reactions of the HMP pathway (Tiwari and Campbell, 1969). An alternate route consisted of triose phosphate condensation by fructose diphosphate aldolase and subsequent conversion to fructose 6-phosphate by fructose diphosphatase (Table X). During growth on glycerol, the appropriate enzymes were available for conversion of triose phosphate to hexose phosphate which was converted oxidatively to pentose phosphate (Table X). Cells grown on lactate or succinate contained the requisite enzymes for hexose phosphate biosynthesis from phosphoenolpyruvate via fructose diphosphate (Table X). Pentose phosphate was presumably formed from hexose phosphate by the nonoxidative reactions of the HMP pathway since 6-phosphogluconate dehydrogenase was not present under these conditions.

Thus PA-1 accomplished gluconeogenesis and pentose phosphate biosynthesis by the usual mechanisms. This was also shown to be the case in P. aeruginosa ATCC 9027 (Tiwari and Campbell, 1969). However, such mechanisms were precluded in P. aeruginosa ATCC 7700 due to the apparent absence of fructose diphosphate aldolase. Furthermore, Ruiz-Amil et al. (1969) have suggested that other routes are likely in P. putida A.3.12. Cells grown on succinate, lactate or acetate had very low levels of glyceraldehyde 3-phosphate dehydrogenase (NAD^+- and $NADP^+$-linked). Thus, in spite of adequate levels of all other

necessary enzymes, triose phosphate levels would severely limit hexose phosphate synthesis. These workers assayed GAP dehydrogenase by reduced pyridine nucleotide oxidation in the presence of 3-phosphoglycerate and excess phosphoglycerate kinase. It should be mentioned that in the present study this activity was assayed by pyridine nucleotide reduction since the reverse reaction consistently gave lower activity with NADPH in spite of the presence of high levels of phosphoglycerate kinase (Table X). Therefore, several unresolved differences regarding gluconeogenesis and pentose phosphate biosynthesis apparently exist among the pseudomonads.

Tiwari and Campbell (1969) noted the presence of both NAD^+- and $NADP^+$-linked GAP dehydrogenase activities in P. aeruginosa but no evidence was presented which would distinguish between a single enzyme nonspecific with respect to pyridine nucleotide coenzyme and two distinct enzymes. The present study has confirmed the presence of both activities and demonstrated the existence of two GAP dehydrogenases, each presumably coenzyme specific (Table X). The $NADP^+$-linked GAP dehydrogenase was present in extracts of cells grown on each substrate, being somewhat higher in glucose and gluconate grown cells. This enzyme was available for both degradation and biosynthesis of GAP and was therefore amphibolic. The NAD^+-linked GAP dehydrogenase was essentially absent from lactate and succinate grown cells and was specifically induced by substrates degraded at least in part via GAP. This enzyme was therefore catabolic. In addition, the NAD^+-linked activity was more unstable than the $NADP^+$-linked activity in crude extracts. Ruiz-Amil et al. (1969) have also noted that glucose and glycerol induced NAD^+-linked GAP dehydrogenase in P. putida A.3.12 and have

suggested that GAP is the inducer.

The pathways of glucose, gluconate and 2-ketogluconate degradation converge at the level of 6-phosphogluconate. This is also the branch point from which the ED and HMP pathways diverge (cf. Figure 2). Control of 6-phosphogluconate formation and consumption at the enzyme level might be expected in view of its central position. Lessie and Neidhardt (1967a) have emphasized the advantages of ATP-controlled glucose 6-phosphate dehydrogenase activity in P. aeruginosa. The possibility of phosphate-controlled glucose 6-phosphate dehydrogenase pyridine nucleotide specificity (Table XII) has been discussed.

The control of synthesis of the enzymes of glucose metabolism was investigated by comparing enzyme levels in extracts of PA-1 grown on glucose, gluconate, glycerol, lactate and succinate. These data are summarized in Table X.

In general, the enzymes of the TP pathway which are involved in converting GAP to pyruvate and the enzymes of the EMP pathway which are involved in hexose phosphate synthesis from GAP appeared to be constitutive and relatively insensitive to changes in growth substrate. This might have been anticipated for the TP pathway enzymes since all except pyruvate kinase were amphibolic.

In contrast, the enzymes specifically involved in catabolism of glucose and related compounds via the ED and HMP pathways were inducible. These included glucose dehydrogenase, gluconate dehydrogenase, glucokinase, glucose 6-phosphate dehydrogenase, gluconokinase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase, KDPG aldolase and NAD^+-linked GAP dehydrogenase. The basal levels of these enzymes were defined by their uninduced levels in extracts of lactate

and succinate grown cells. It should be noted that gluconokinase, 6-phosphogluconate dehydrogenase and NAD^+-linked GAP dehydrogenase were extremely low or undetectable in these extracts.

Growth on glucose induced high levels of glucokinase, glucose 6-phosphate dehydrogenase, gluconate dehydrogenase, gluconokinase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase, KDPG aldolase and NAD^+-linked GAP dehydrogenase. Glucose dehydrogenase was only weakly induced. The high level of gluconate dehydrogenase would appear to be of questionable value to glucose catabolism in view of the low glucose dehydrogenase activity. Similar arguments apply to gluconokinase which must also compete with a ten-fold excess of gluconate dehydrogenase for a limited supply of gluconate. These results confirmed and extended those of other studies of glucose induction of the glucose catabolic enzymes of P. aeruginosa (von Tigerstrom and Campbell, 1966; Ng and Dawes, 1967; Lessie and Neidhardt, 1967a; Tiwari and Campbell, 1969).

Growth on gluconate induced glucose dehydrogenase, glucokinase, glucose 6-phosphate dehydrogenase, gluconate dehydrogenase, gluconokinase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase, KDPG aldolase and NAD^+-linked GAP dehydrogenase. The formation of glucose dehydrogenase and glucokinase was gratuitous unless excessive amounts of hexose phosphate were accumulating and being converted to glucose by a phosphatase. This seemed unlikely since hexose phosphate synthesis from GAP by reversal of the EMP sequence would be limited by fructose diphosphate aldolase which would have to compete with a greater than ten-fold excess of GAP dehydrogenase (NAD^+- and $NADP^+$-linked). Hexose phosphate synthesis via the HMP pathway would appear

more likely but the level of 6-phosphogluconate dehydrogenase was significantly lower than that found in extracts of glucose grown cells and must also compete with 6-phosphogluconate dehydrase. The lower 6-phosphogluconate dehydrogenase level is also seen in the data of Lessie and Neidhardt (1967a) and may be related to the radiorespirometric observation that P. aeruginosa degraded glucose via the ED and HMP pathways but degraded gluconate solely via the ED pathway (Wang et al., 1959).

Surprisingly, growth on glycerol induced glucose dehydrogenase, glucokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase, KDPG aldolase and NAD^+-linked GAP dehydrogenase. Gluconate dehydrogenase was only weakly induced and gluconokinase was absent. Glycerol induced the highest level of glucose dehydrogenase observed. These results confirmed and extended those of Lessie and Neidhardt (1967a). As in the case of growth on gluconate, synthesis of glucose dehydrogenase and glucokinase during growth on glycerol was gratuitous. These results strongly suggested that significant amounts of glycerol were being metabolized via hexose phosphate although there was no obvious need for such an arrangement. However, the fact that most glucose mutants were also glycerol negative indicated that such a relationship might be obligatory. Hexose phosphate would presumably be formed from GAP by reversal of the EMP sequence. It is interesting that Neidhardt's P. aeruginosa ATCC 7700 which lacks fructose diphosphate aldolase also induced the glucose catabolic enzymes during growth on glycerol.

A direct role for glucose dehydrogenase on the main pathway of glucose metabolism was precluded by the regulation of its synthesis.

Glucose dehydrogenase was lowest in glucose grown cells, intermediate in gluconate grown cells and highest in glycerol grown cells. This was consistent with the view that direct hexose oxidation represents a mechanism for continued glucose consumption under conditions where the main pathway is saturated (Sokatch, 1969). This might be of survival value to the organism in nature. It follows that glucose dehydrogenase induction should be regulated by the level of main pathway intermediates rather than by glucose. This concept was supported by the fact that glucose did induce high levels of glucose dehydrogenase under conditions where intermediates were expected to accumulate (Table XI). Induction of glucose dehydrogenase by gluconate and glycerol presumably reflected accumulation of the inducing intermediate.

The mode of gluconate dehydrogenase induction was clearly different from that of glucose dehydrogenase. Gluconate dehydrogenase was lowest in cells grown on glycerol, intermediate in cells grown on gluconate and highest in cells grown on glucose. This behavior was more consistent with a possibly significant role for gluconate dehydrogenase in gluconate metabolism. Such a role would suggest that gluconate was the inducer for gluconate dehydrogenase. However, several observations indicated that this may not be so. Growth on glucose induced higher levels of gluconate dehydrogenase than did growth on gluconate (except in the presence of nutrient broth, see Table XI). Comparison of the levels of gluconate dehydrogenase and glucose dehydrogenase in cells grown on or exposed to glucose showed that the level of gluconate dehydrogenase was not directly related to the ability to form gluconate from glucose. Thus, the possibility of gluconate dehydrogenase induction by an intermediate could not be eliminated but the

observations suggesting this were not compelling either.

Fraenkel and Horecker (1964) showed that S. typhimurium possessed an inducible ED pathway which functioned in gluconate but not glucose catabolism. Gluconate specifically induced gluconokinase and 6-phosphogluconate dehydrase whereas glucose did not. An identical situation was demonstrated in E. coli (Fraenkel and Levisohn, 1967; Zablotny and Fraenkel, 1967). Eisenberg and Dobrogosz (1967) confirmed and extended these observations to Aerobacter aerogenes and suggested that gluconate was also the inducer for the ED pathway in P. fluorescens.

Gluconate does not appear to be the inducer for the ED pathway in PA-1. On the contrary, there was evidence that the main pathway enzymes may also be induced by intermediates. Gratuitous synthesis of glucokinase during growth on gluconate and glycerol has been noted above. Furthermore, growth on glycerol also induced the entire sequence of main pathway enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase, KDPG aldolase and NAD^+-linked GAP dehydrogenase). These results were more readily interpreted in terms of induction by intermediates.

Synthesis of the inducible enzymes of glucose metabolism did not appear to be coordinate. Pair-wise comparison of enzyme levels in extracts of PA-1 grown on various substrates did not indicate coordinate synthesis of any of these enzymes (Table X). Similar conclusions were reached on examining the data available in the literature (von Tigerstrom and Campbell, 1966; Lessie and Neidhardt, 1967a; Tiwari and Campbell, 1969). However, these data were not obtained for this purpose and consequently the values tend to be rather extreme. An

investigation of enzyme levels under a variety of conditions permitting a continuous variation of enzyme levels would be desirable.

Before considering the mutant enzyme data and their implications for control of the synthesis of the inducible glucose catabolic enzymes, it is important to note the limitations of these data. Initially, enzyme profiles of several mutants were determined in extracts of cells grown on nutrient broth containing 0.5 per cent glucose. However, this method was not applicable to those strains which lysed in glucose-supplemented nutrient broth (Table V). Glucose was also known to cause severe inhibition of growth of certain mutants on various substrates including histidine (Table IV). In an attempt to induce the glucose enzymes of each mutant under comparable conditions, the following procedure was adopted. The cells were grown on lactate minimal medium, washed and then exposed to various inducing substrates for four hours. The cells were capable of only limited metabolism of the inducing substrate (except 707, 718, and 732 induced with glycerol). Therefore, the carbon and energy for induced enzyme synthesis must be supplied from cellular material. P. aeruginosa does not form storage products (MacKelvie, et al., 1968) and apparently degrades endogenous protein as its reserve material (Warren et al., 1960). Thus, it seems likely that acquisition of the capacity to form the inducible glucose catabolic enzymes is at the expense of ribosomes (Gronlund and Campbell, 1965) and energy generating systems which are required for enzyme synthesis. In addition, glucose and related compounds may retard the generation of required carbon and energy by catabolite inhibition and catabolite repression. Furthermore, the inducible glucose catabolic enzymes may be subject to catabolite repression by the end products of

amino acid degradation (Ng and Dawes, 1967). The situation is further complicated by the possibility that intermediates may be the true inducers and enzyme synthesis is noncoordinate.

Comparison of glycerol-induced and glycerol-grown enzyme profiles for 707, 718 and 732 illustrated these problems (Table XIV). Induction yielded generally lower enzyme levels than did growth. In each case, the relative levels of glucose dehydrogenase and gluconate dehydrogenase were reversed. The four hour induction period was not adequate for induction of 6-phosphogluconate dehydrogenase which appeared only during growth on glycerol.

Several other factors should also be considered. Accumulation of some intermediates could inhibit further enzyme synthesis. Lysis in glucose-supplemented nutrient broth apparently resulted from accumulation of KDPG due to the absence of KDPG aldolase in 720 and related strains (Table V, Table VII, and Table XVIII). Accumulation of KDPG may have caused the extremely low levels of gluconokinase and 6-phosphogluconate dehydrogenase detected in 720 and 755 (Table XVIII). The recognition of endogenous induction of NAD^+-linked GAP dehydrogenase in mutants of the 760 group and 720 group altered conclusions regarding its inducibility by various substrates in those mutants (Table XV and Table XVIII). A similar effect may be responsible for the uniformly high levels of gluconokinase observed in mutants of the 760 group induced with each substrate. The reason for the uniformly high levels of glucose 6-phosphate dehydrogenase seen in 755 but not 720 was not clear but may also be a related phenomenon. The fact that the endogenous NAD^+-linked GAP dehydrogenase was partially degraded during induction with gluconate and glycerol illustrated the difficulty

in assigning genetic lesions to enzymes whose basal levels were low or undetectable in the wild type (gluconokinase, 6-phosphogluconate dehydrogenase and NAD^+-linked GAP dehydrogenase). In such cases, absence of activity could be due either to failure to induce and degradation of basal enzyme levels or direct inactivation of the gene product by the mutation.

In general, the enzyme data for mutants supported the concept that the enzymes of direct hexose oxidation are induced by intermediates. All mutants except 730 and 736 retained the ability to synthesize glucose dehydrogenase and gluconate dehydrogenase. In each of these strains, with the possible exceptions of 773 and 777, exposure to glucose induced glucose dehydrogenase (Tables XIV through XVIII). Gluconate was a poor inducer of gluconate dehydrogenase in these strains with the exceptions of 720 and 755. More significantly, exposure to glycerol induced the highest levels of gluconate dehydrogenase in these mutants except for 734. However, as noted previously, L-α-glycerophosphate dehydrogenase could not be detected in the extract of glycerol-induced 734. These observations were consistent with the view that the enzymes of direct hexose oxidation are responsive to accumulated intermediates and that these intermediates were generated from glycerol as well as glucose and gluconate.

It was interesting that exposure to glycerol, which is degraded via GAP, induced NAD^+-linked GAP dehydrogenase only in 707, 718 and 732 which were able to grow on glycerol. Disregarding 734 and 736, which appeared to be deficient in L-α-glycerophosphate dehydrogenase, exposure to glycerol failed to induce this activity in all other mutants. Furthermore, glucose induction of 732 caused a six-fold increase in

NAD^+-linked GAP dehydrogenase. Inspection of the enzyme profile for 732 showed that there was no way to form GAP from glucose under the conditions of induction. Similar results were obtained for glucose-induced 720. Thus, GAP appears to be neither necessary nor sufficient for induction of NAD^+-linked GAP dehydrogenase.

The assignment of inducer roles to various metabolites based solely on enzyme data is dubious since enzyme levels do not necessarily reflect metabolite concentrations. However, the response of NAD^+-linked GAP dehydrogenase to induction by glucose in 720 and 755 deserves further consideration (Table XVIII). Both strains had similar high endogenous levels of this activity in lactate-grown cells. Induction with gluconate or glycerol led to partial degradation of the enzyme in both strains. Induction with glucose caused a two-fold increase of NAD^+-linked GAP dehydrogenase in 720 but merely maintained the endogenous level in 755. The only other difference between these strains was that 720 had very low glucose 6-phosphate dehydrogenase under all conditions of induction whereas 755 had very high levels under the same conditions. It seems reasonable that 720 would accumulate higher levels of glucose 6-phosphate than 755. This suggests that glucose 6-phosphate is the inducer for NAD^+-linked GAP dehydrogenase.

The available data do not warrant further speculation on the identity of the metabolites responsible for induction of specific enzymes or groups of enzymes. This problem will require further investigation. Attention should be focused on the roles of glucose 6-phosphate and 6-phosphogluconate since intermediates appear to be inducers but KDPG is not involved (Table XIV). The intracellular concentrations of each intermediate should be determined and compared with

the enzyme profile under various conditions. The possible activity of phosphatases should also be examined. A technique for altering the permeability of P. aeruginosa to phosphorylated compounds has recently been reported (Kay and Gronlund, 1969). This may allow direct tests of intermediates as inducers.

Dependence of Glycerol Degradation on Glucose Metabolism

Cowen (1968) established that PA-1 degraded glycerol via L-α-glycerophosphate and dihydroxyacetone phosphate to GAP. Glycerol kinase and L-α-glycerophosphate dehydrogenase were inducible and, on the basis of substrate removal data, the system appeared to be subject to catabolite inhibition and possibly catabolite repression by glucose. Tsay (1971) demonstrated an inducible transport system for glycerol in PA-1. The transport system did not appear to be repressed by glucose. Determination of glycerol kinase and L-α-glycerophosphate dehydrogenase in extracts indicated that neither was repressed significantly by glucose.

Lessie and Neidhardt (1967a) observed that glycerol induced high levels of the main pathway glucose catabolic enzymes (glucokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase and KDPG aldolase). The present study has confirmed and extended those observations in PA-1 as previously discussed. However, these results were puzzling since there was no apparent need for such an arrangement (cf. Figure 2). GAP could be catabolized via the TP pathway and gluconeogenesis and pentose phosphate synthesis could proceed via reversal of the EMP sequence and the nonoxidative reactions of the HMP pathway, respectively. However, the

facts that glycerol induced the glucose catabolic enzymes and that most glucose mutants were also glycerol negative indicated that an obligatory dependence of glycerol catabolism on glucose metabolism might exist. Such an arrangement could account for the apparent lack of catabolite repression, the low levels of glycerol kinase and L-α-glycerophosphate dehydrogenase in fully induced cells and the slow growth of PA-1 on glycerol; that is, glycerol catabolism could be under a constant state of self-imposed catabolite repression due to a unique dependence on glucose metabolism.

Various alternative explanations for the high frequency of glycerol negative glucose mutants were considered and subsequently eliminated. As discussed previously, reversion analysis indicated that failure to grow on glucose and glycerol was due to the same defect but that growth on glycerol could be restored by a further mutation which did not restore growth on glucose (Table VIII). The TP and EMP pathways were intact; none of the mutants lacked pyruvate kinase (Table XIII). These results led to the conclusion that glycerol catabolism does depend on steps in glucose metabolism prior to GAP, the point at which these pathways converge.

Growth of PA-1 on glycerol failed to induce gluconate dehydrogenase and gluconokinase, indicating that these enzymes were not relevant to growth on glycerol (Table X). Arguments have been presented that induction of glucose dehydrogenase and glucokinase during growth on glycerol was gratuitous. Neither 6-phosphogluconate dehydrase nor KDPG aldolase was required for growth on glycerol (Table XIV). As noted above, glycerol induced NAD^+-linked GAP dehydrogenase only in those strains able to grow on glycerol (707, 718, and 732) and

6-phosphogluconate dehydrogenase activity appeared only during growth on glycerol. Furthermore, the ability to grow on glycerol appeared to be limited by the level of 6-phosphogluconate dehydrogenase in 732 (Table III and Table XIV). Mutants of the 760 group were similar to those of the 707 group except that they failed to grow on glycerol. This was presumably due to 6-phosphogluconate dehydrogenase deficiency (Table XV).

The following model relating glycerol catabolism to glucose metabolism can be proposed on the basis of the present study. Glycerol is degraded to GAP. Although $NADP^+$-linked GAP dehydrogenase is present, there is a dearth of $NADP^+$ since cells tend to keep this pyridine nucleotide reduced (Horecker, 1965). Oxidation of NADPH was quite slow in crude extracts of PA-1. Thus, the NAD^+-linked GAP dehydrogenase is required for efficient catabolism of GAP. However, this enzyme is not induced directly by GAP; induction requires conversion of glycerol to glucose 6-phosphate, which induces NAD^+-linked GAP dehydrogenase. Hexose phosphate does not accumulate in PA-1 due to the presence of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase and KDPG aldolase. Mutants lacking 6-phosphogluconate dehydrase or KDPG aldolase will accumulate intermediates unless 6-phosphogluconate dehydrogenase is formed as in the 707 group. Failure to remove accumulated intermediates presumably accounts for the inability of mutants containing all other relevant enzymes to grow on glycerol.

The fact that type I revertants selected on glycerol (and unable to grow on glucose) were obtained from such strains suggests that this problem can be overcome by second site mutation. The model predicts

several possible mechanisms for restoring growth on glycerol independent of growth on glucose. Synthesis of NAD^+-linked GAP dehydrogenase could become constitutive thus obviating the need for extensive conversion of GAP to hexose phosphate. If 6-phosphogluconate were the main cause of inhibition, loss of glucose 6-phosphate dehydrogenase would permit induction of NAD^+-linked GAP dehydrogenase and prevent accumulation of inhibitor. Mutations altering the control of 6-phosphogluconate dehydrogenase synthesis could restore sufficient levels of this enzyme to dispose of accumulated intermediates. In fact, it is conceivable that mutants of the 707 group are actually double mutants lacking 6-phosphogluconate dehydrase and having altered control of 6-phosphogluconate dehydrogenase. The situation regarding 720 and 755 is somewhat different. These strains lacked KDPG aldolase and showed trace 6-phosphogluconate dehydrogenase activity following induction with glycerol (Table XVIII). Their failure to grow on glycerol is easily rationalized by the deleterious effects of KDPG accumulation. Thus, type I revertants of these strains selected on glycerol might be expected to have lost 6-phosphogluconate dehydrase.

Although the model is speculative, it does make specific predictions which can be tested experimentally. Enzyme analysis of type I glycerol revertants will certainly help to clarify further the relationship between glycerol and glucose metabolism. PA-1 grows rather slowly on glycerol and a similar analysis of fast growing mutants would also be helpful.

Genetic Considerations and Linkage of the Glucose Loci

The mutants isolated and characterized in the present study were selected for their inability to grow on glucose, or gluconate in the cases of 773 and 777 (Table I). Three pathways diverge from glucose and converge at 6-phosphogluconate (cf. Figure 2). Thus, the isolation procedure was biased in favor of mutants lacking 6-phosphogluconate dehydrase and/or KDPG aldolase since a single lesion in any of the three convergent pathways would not prevent growth on glucose or gluconate by an alternate route. It was not surprising then that each mutant failed to grow on glucose, gluconate and 2-ketogluconate (Table II). This would be expected of mutants lacking either or both of these two key ED pathway enzymes.

The mutants were separated into two groups on the basis of lysis in glucose-supplemented nutrient broth (Table V). Qualitative transduction tests led to precisely the same separation (Table VII). Although genetic heterogeneity was evident within these two large groups, transduction tests provided little useful information for subgrouping the mutants since the tests were not quantitative. The group I mutants (nonlysing) were subdivided on the basis of growth on glycerol; only strains 707, 718 and 732 were able to grow on glycerol (Table III).

The most useful information was obtained by enzyme analysis. Group I mutants all lacked at least 6-phosphogluconate dehydrase (Tables XIV through XVII). Representative mutants from group II, which is composed of glycerol negative strains exhibiting the lysis phenomenon, lacked KDPG aldolase (Table XVIII). This was consistent with the

mutant isolation methods and the inability of these strains to grow on glucose, gluconate and 2-ketogluconate.

Heterogeneity within group I and group II was apparent upon comparison of mutant enzyme profiles (Tables XIV through XVIII). Mutants with similar enzyme profiles were placed in the same subgroup (Table VII).

Mutants 707, 718 and 732 lacked 6-phosphogluconate dehydrase; 6-phosphogluconate dehydrogenase was detected only after growth on glycerol (Table XIV). Mutants 760, 773, 777 and 728 lacked 6-phosphogluconate dehydrase and 6-phosphogluconate dehydrogenase (Table XV). Mutants 734 and 738 lacked both these enzymes and also gluconokinase (Table XVI). Finally, mutants 730 and 736 lacked 6-phosphogluconate dehydrase, 6-phosphogluconate dehydrogenase, gluconokinase, glucose dehydrogenase and gluconate dehydrogenase (Table XVII). However, 730 and 736 were not identical since 736 was also impermeable to glucose (Table VI and Figure 9). Within each of these subgroups, differences were noted in the mutant enzyme profiles and were presumably related to the observed genetic heterogeneity within certain subgroups. Thus, the mutants of group I could be arranged in a series of subgroups showing progressively more complex enzyme lesion patterns.

Likewise, mutants of group II showed genetic and enzymatic differences. Mutants 720 and 745 did recombine with other members of the group (Table VII). The difference in glucose 6-phosphate dehydrogenase levels between 720 and 755 has been noted (Table XVIII). Partial enzyme analysis of 745 revealed high glucose 6-phosphate dehydrogenase similar to 755.

The cause of the multiple enzyme lesions observed in many group I mutants was not entirely clear. As noted previously, the basal levels of gluconokinase and 6-phosphogluconate dehydrogenase were very low or undetectable in PA-1. The absence of these activities was probably the result of a control phenomenon rather than a direct mutational alteration of the corresponding structural genes since these mutants also lacked 6-phosphogluconate dehydrase and glucose positive revertants have been noted for each strain except 730 and 736. This eliminated extensive deletion of contiguous structural genes as a possible explanation and argued against independent mutations, at least in the case of 6-phosphogluconate dehydrogenase, assuming that this enzyme is necessary for normal growth on glucose.

The known mechanisms by which point mutations could cause pleiotropic negative effects include regulator gene mutations generating super-repressors in negative control systems or inactive activators in positive control systems, promoter mutations and polar structural gene mutations. Only the latter two mechanisms require contiguity of the affected genes since their effects are a consequence of transcription and translation of polycistronic mRNA. Since 6-phosphogluconate dehydrase and 6-phosphogluconate dehydrogenase did not appear to be coordinately controlled and gluconokinase was clearly subject to a different control mechanism (Table X), promoter and polar mutations seemed unlikely as the cause of the pleiotropic deficiencies. Promoter and regulator gene mutations were also difficult to reconcile with the fact that mutants can lack 6-phosphogluconate dehydrogenase or both 6-phosphogluconate dehydrogenase and gluconokinase.

Epstein and Beckwith (1968) have noted that frequent isolation of pleiotropic negative regulatory mutants is characteristic of positively controlled systems. However, it should be noted that sequential induction could lead to similar results. A structural gene lesion which prevents formation of the inducer of another enzyme or group of enzymes would show pleiotropic negative effects. Furthermore, accumulation of intermediates preceding an enzyme lesion might repress the synthesis of other enzymes.

Further study will be required to resolve this problem. Growth studies and enzyme analysis of revertants selected on several substrates should be undertaken to prove that the pleiotropic effects are due to a single point mutation. Determination of the kinetics of induced glucose catabolic enzyme formation might clarify the possible roles of intermediates in induction and repression, especially if correlated with the intracellular concentrations of the intermediates. Further analysis of this system would be greatly facilitated in a strain specifically lacking glucose dehydrogenase and gluconate dehydrogenase since the three convergent pathways would then be independent. However, isolation of such a mutant poses some difficult selection problems.

Strains 730 and 736 were apparently deletion mutants. These strains exhibited the most extensive pattern of enzyme lesions as noted above. Glucose positive revertants have never been observed for either strain, even after NTG mutagenesis in the case of 736 (Table VIII).

Whether or not gluconokinase and 6-phosphogluconate dehydrogenase were included in the deletion can not be established from the data presently available. Enzyme analysis of glycerol positive revertants

could demonstrate that either or both are not. Strains with known point mutations in either structural gene were not available for transduction tests with the deletion mutants. Furthermore, such strains will probably be difficult to construct due to the non-independence of the three convergent pathways.

Glucose dehydrogenase and gluconate dehydrogenase have been tentatively included in the deletion since the trace activities seen in some extracts were probably due to nonspecific endogenous dye reduction. The very close linkage of the corresponding structural genes suggested by simultaneous deletion has no significance in terms of coordinate control of these enzymes since they were clearly subject to different control mechanisms (Table X). It was evident that the 6-phosphogluconate dehydrase structural gene must have been only partially deleted in 730 and 736 since a few transductants were obtained with known 6-phosphogluconate dehydrase mutants (Table VII). This defined one end of the deletion. Strain 730 was permeable to glucose whereas 736 was not. Thus, the deletion in 736 was apparently more extensive than in 730. The glucose permeability marker defined the other end of the deletion.

Therefore, several of the structural genes coding for glucose catabolic functions appeared to be clustered. This was of doubtful significance in terms of control since several of the enzymes coded by the proposed cluster were not induced coordinately. Furthermore, clustering was not complete since the structural genes for 6-phosphogluconate dehydrase and KDPG aldolase were not linked (Table VII). Thus, it can be tentatively concluded that the distribution of genes coding for glucose catabolic functions is best described as

scattered gene clusters. This is consistent with the results of similar investigations of other pathways in Pseudomonas (Holloway, 1969).

Control of Histidase Synthesis

Lessie and Neidhardt (1967b) have shown that histidase, urocanase and the formiminoglutamate-degrading enzyme, which mediate the first, second and fourth steps of histidine catabolism, respectively, are inducible and are subject to catabolite repression by a variety of substrates including succinate and glucose. Convincing evidence was presented that urocanate, the first intermediate, is the true inducer rather than histidine. This was recently proved using a histidase deficient mutant (Newell and Lessie, 1970). On the basis of growth and substrate removal data, Cowen (1968) concluded that histidine catabolism in PA-1 was inducible by either histidine or urocanate but was not subject to catabolite inhibition or catabolite repression by glucose.

In the present study, the control of histidine catabolism in PA-1 was examined by assaying histidase in whole cells (Table XIX). Histidase was induced during growth on either histidine or urocanate. The presence of glucose in the growth medium repressed histidine- and urocanate-induced histidase formation to the same extent, approximately 75 per cent. The residual histidase level and lack of catabolite inhibition by glucose account for the mistaken conclusion that histidine catabolism in PA-1 was insensitive to catabolite repression.

Histidase synthesis was also repressed by other substrates. Succinate caused the most severe repression of histidase formation (85 per cent). The severity of succinate repression may be partially

due to a sequential feedback inhibition of histidase activity by succinate which has been described in P. putida (Hug et al., 1968). Succinate inhibited urocanase which caused urocanate to accumulate from histidine via histidase. The histidase was in turn subject to product inhibition by the accumulated urocanate. However, the physiological significance of this mechanism in terms of regulation of enzyme synthesis is questionable since urocanate also appears to be the true inducer in P. putida (Wheelis and Stanier, 1970).

Gluconate repression of histidase synthesis was comparable to that exerted by glucose. Catabolite repression of histidase by lactate afforded an example of an exception to the general rule that readily utilized substrates are more repressive. Lactate was an excellent substrate for PA-1 but produced only a mild repression of histidase (45 per cent). Glycerol was only weakly repressive, as expected from its inability to support rapid growth of PA-1.

Catabolite repression of histidase by glucose and gluconate in PA-1 was fully confirmed in experiments where the differential rate of histidase synthesis was determined following addition of glucose or gluconate to cells growing on histidine (Figure 10). Glucose and gluconate repressed the differential rate of histidase synthesis approximately 70 per cent, based on comparison with the control. It should be noted that these experiments were performed under conditions known to elicit transient repression in E. coli. No such effect was observed in this system. If transient repression depends on rapid entry of the added substrate and a functional EI of the phosphoenolpyruvate phosphotransferase system as suggested by Tyler and Magasanik (1970), then the absence of transient repression following addition of glucose or

gluconate was not surprising since glucose transport is inducible in P. aeruginosa (Tiwari and Campbell, 1969; Phibbs and Eagon, 1970). Furthermore, attempts to demonstrate the phosphoenolpyruvate-dependent system in P. aeruginosa have been unsuccessful (Phibbs and Eagon, 1970).

In similar experiments, the simultaneous addition of 1 mM cAMP did not appear to relieve catabolite repression of histidase by glucose or gluconate in PA-1. However, these results can not be considered conclusive since a higher concentration may be required. In addition, Pseudomonas may require a treatment to alter the permeability of the cells to this cyclic nucleotide (Perlman and Pastan, 1968). In view of the central role of cAMP in regulation of the synthesis of catabolite sensitive enzymes in the enterics, further investigations of cAMP effects in Pseudomonas should be undertaken.

During growth studies of the effect of glucose on histidine catabolism in various glucose mutants, it was noted that the severity of the inhibition depended on whether or not the cells were induced for histidine catabolism prior to exposure to glucose. Addition of glucose to 707 cells just beginning to grow on histidine (i.e., induced) resulted in linear growth (Figure 4). Linear growth was interpreted as resulting from progressive dilution by cell division of the carbon- and energy-yielding histidine catabolic system due to catabolite repression. However, when uninduced cells were subcultured into a mixture of histidine and glucose, a more severe growth inhibition occurred. Mutant 707 was shown to convert glucose nearly quantitatively to an intermediate tentatively identified as gluconate which appeared in the medium (Figure 8). Gluconate, like glucose, caused linear growth of induced

707 cells and severe growth inhibition of uninduced 707 cells (Figure 7).

These results suggested that glucose and gluconate could exert another effect on histidine catabolic enzyme formation in addition to catabolite repression and that this was an indirect effect on the induction process exerted through prevention of inducer entry. This was supported by the observation that very low concentrations of the intermediate accumulated from glucose by 707 (gluconate) caused uninduced 707 cells to experience a growth lag on histidine which was proportional to the concentration of intermediate present (Figure 5). The fact that subsequent growth was normal indicated that the low concentration of intermediate had no further effect once induction took place.

The apparent inhibition of histidine transport was most clearly demonstrated by comparing the effect of glucose on growth of uninduced and induced 736 on histidine. Strain 736 does not metabolize glucose and is impermeable to it, yet growth of uninduced 736 cells in a mixture of histidine and glucose was inhibited (Table IV). Therefore, glucose must exert a direct effect outside the cell. However, addition of glucose to induced 736 cells had essentially no effect on growth on histidine (Figure 13a).

All these data suggest that glucose (or gluconate) can prevent entry of histidine in sufficient quantity to bring about rapid induction of histidase in uninduced cells, but does not appreciably affect histidine transport in induced cells. These observations may indicate that PA-1 possesses two transport systems for histidine, one of which is constitutive and functions in transport of histidine at the lower

rates required for protein synthesis. A histidine transport system of this type which is partially inhibited by glucose has been reported in S. typhimurium (Ames, 1964). A second transport system, required for uptake at rates sufficient to support growth on histidine as the sole source of carbon and energy, would be expected to be inducible. Newell and Lessie (1970) have recently demonstrated a histidine transport system in citrate grown P. aeruginosa cells. This system was increased approximately four-fold by growth on a mixture of citrate and histidine. Histidine rather than urocanate apparently induces this additional transport activity since it also occurs in a mutant lacking histidase. Inhibition of the constitutive system by glucose would prevent or delay accumulation of sufficient histidine to induce a transport system not subject to inhibition by glucose.

Urocanate can be detected spectrophotometrically in the supernatant from a culture of PA-1 growing on histidine. This probably accounts for the presence of a stimulating factor in the challenged filtrate since urocanate appears to be the true inducer in PA-1 (Figure 6). Thus, it seems likely that the growth lag described above represents the time required to transport and convert enough histidine to urocanate via the basal histidase level to effect induction of the histidine catabolic system.

The identity of the glucose catabolite which serves as effector of catabolite repression of histidase was investigated in glucose mutants with the appropriate enzyme lesions. The effector was presumably an intermediate readily derivable from either glucose or gluconate since the two compounds were equally efficient in eliciting catabolite repression of histidase in PA-1. Strains 707, 730 and 736 were

selected on the basis of their enzyme lesions and permeability characteristics which have been discussed previously. The differential rate of histidase synthesis was determined for each strain following addition of glucose or gluconate to cells just beginning to grow on histidine.

Strain 707 was expected to accumulate 6-phosphogluconate and all preceding intermediates from glucose; exposure to gluconate should allow accumulation of 2-ketogluconate, 2-keto-6-phosphogluconate and 6-phosphogluconate. Addition of either glucose or gluconate caused the usual linear growth and repressed the initial differential rate of histidase synthesis approximately 65 per cent in 707 growing on histidine (Figures 11a and 11b). This was quite comparable to the 70 per cent repression exerted by glucose and gluconate in PA-1. Thus, the effector was produced efficiently from either glucose or gluconate in 707. Possible candidates for the effector role included gluconate, 2-ketogluconate, 2-keto-6-phosphogluconate and 6-phosphogluconate.

Strain 730 was expected to accumulate only glucose 6-phosphate and 6-phosphogluconate (inefficiently) from glucose. Although gluconate may permeate these cells, it can not be further metabolized. Addition of glucose caused a slight inhibition of growth and repressed the differential rate of histidase synthesis 27 per cent in 730 growing on histidine; addition of gluconate had no effect on growth or histidase synthesis (Figures 12a and 12b). Thus, the basal enzyme levels of 730 were able to generate the effector inefficiently from glucose. The only intermediate common to 707 and 730 under conditions of catabolite repression was 6-phosphogluconate.

Strain 736 had the same enzyme profile as 730 but was also impermeable to glucose and should not accumulate intermediates from either glucose or gluconate. Addition of either glucose or gluconate had no effect on growth or histidase synthesis in 736 growing on histidine (Figures 13a and 13b).

These results demonstrate that glucose and gluconate must enter the cell and be metabolized to effect catabolite repression of histidase. The effector of this repression has been tentatively identified as 6-phosphogluconate. Further evidence supporting this conclusion will require correlation of the intracellular concentration of 6-phosphogluconate with the degree of repression. As noted previously, it may be possible to alter the permeability of the cells sufficiently to test the repressive effects of phosphorylated intermediates directly. Any possible role of phosphatases should also be examined. Construction of a strain lacking glucose dehydrogenase and glucose 6-phosphate dehydrogenase would also allow a definitive test of 6-phosphogluconate as the effector.

Lessie and Neidhardt (1967b) have concluded that the effector of catabolite repression of the histidine catabolic system is a nitrogenous metabolite closely related to succinate since succinate repression was relieved when histidine was the sole nitrogen source. To the extent that linear growth reflects catabolite repression, this does not appear to be the case in PA-1 since addition of glucose to 707 growing on histidine as sole carbon, nitrogen and energy source resulted in linear growth. However, this should be more thoroughly investigated by determining the differential rate of histidase synthesis. It is conceivable that more than one metabolite can effect repression of

histidase synthesis. Indeed, mutants 707, 730 and 736 were shown to be subject to normal catabolite repression by succinate and lactate (Table XX). It is not known whether succinate and lactate effect repression via 6-phosphogluconate. The recent isolation of a mutant of PA-1 tentatively characterized as lacking triose phosphate isomerase (F. White, personal communication) may permit resolution of these questions.

The potential of this system has not been fully exploited in the present study. Glucose has been observed to inhibit growth of 707 on glycerol. Meganathan (1970) has found that glucose inhibited growth of 707 on allantoin in a manner similar to glucose inhibition of growth on histidine. The inhibition was apparently due to severe repression of synthesis of ureidoglycolate synthetase. The severe inhibition of growth of 707 on various substrates allows the direct selection of relieved mutants which are still glucose negative. This may provide a means for isolating control mutants in any pathway subject to glucose inhibition in 707 or other glucose mutants.

CHAPTER V

SUMMARY AND CONCLUSIONS

PA-1 lacked a catabolically functional EMP pathway due to the absence of phosphofructokinase. The appropriate enzymes for hexose phosphate biosynthesis from phosphoenolpyruvate via reversal of the TP and EMP pathways were present. Pentose phosphate was presumably formed from hexose phosphate by reversal of the nonoxidative portion of the HMP pathway when 6-phosphogluconate dehydrogenase was not present.

The enzymes of direct hexose oxidation, glucose dehydrogenase and gluconate dehydrogenase, did not appear to play a major role in the catabolism of glucose. Glucose was neither necessary nor sufficient for induction of glucose dehydrogenase. Growth on glycerol yielded the highest levels of glucose dehydrogenase. The levels of these enzymes in extracts of wild type and mutant cells grown or induced under various conditions indicated that glucose dehydrogenase and gluconate dehydrogenase were induced by different intermediates of glucose metabolism. This would be consistent with the view that direct hexose oxidation represents a mechanism for continued glucose metabolism under conditions where the main pathways are saturated.

Glucokinase, glucose 6-phosphate dehydrogenase, gluconokinase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase, KDPG aldolase and the NAD^+-linked GAP dehydrogenase were inducible by either glucose or gluconate. Glycerol also induced all these enzymes except

gluconokinase. There was no evidence of coordinate synthesis within this group of enzymes. Gratuitous synthesis of glucokinase during growth on glycerol and gluconate indicated that this enzyme may be product-induced; glycerol induction of the glucose catabolic enzymes suggested that other main pathway enzymes may also be induced by intermediates.

The presence of two GAP dehydrogenases was demonstrated. The $NADP^+$-linked enzyme was amphibolic. The NAD^+-linked enzyme was catabolic and inducible by substrates degraded at least in part via GAP. However, GAP was neither necessary nor sufficient for induction. Enzyme data from two mutants indicated that glucose 6-phosphate may be the true inducer.

The fact that glycerol induced the glucose catabolic enzymes suggested that glycerol catabolism may depend on glucose metabolism. This was supported by the observation that most glucose negative mutants were also glycerol negative. Reversion analysis indicated that the double defect was due to a single lesion. Several of these strains were known to possess the appropriate glycerol catabolic enzymes. Neither 6-phosphogluconate dehydrase nor KDPG aldolase was required for growth on glycerol. The following model was proposed to explain the dependence of glycerol catabolism on glucose metabolism. Glycerol is metabolized to GAP but further catabolism requires the NAD^+-linked GAP dehydrogenase. This enzyme is induced by glucose 6-phosphate; therefore, glycerol must also be converted to hexose phosphate to effect the necessary induction of GAP dehydrogenase and thus it coincidentally induces the glucose catabolic enzymes.

All glucose mutants lacked at least 6-phosphogluconate dehydrase or KDPG aldolase as expected from their failure to grow on glucose, gluconate and 2-ketogluconate. Growth of mutants lacking KDPG aldolase on glucose-supplemented nutrient broth resulted in lysis which was apparently due to accumulation of KDPG. The reason for the high frequency of apparent pleiotropic negative mutants was not clear but may be related to induction and/or repression by intermediates of glucose metabolism. The structural genes for 6-phosphogluconate dehydrase and KDPG aldolase were not linked. Based on the enzyme lesions and glucose permeability characteristics of two presumptive deletion mutants, the structural genes for a component of the glucose transport system, glucose dehydrogenase, gluconate dehydrogenase and 6-phosphogluconate dehydrase may be clustered. However, such a cluster would have no significance in terms of control of enzyme synthesis since the latter three activities were not synthesized coordinately with each other.

Histidase was inducible by histidine or urocanate and subject to various degrees of catabolite repression by a variety of compounds. No transient repression of histidase was observed following addition of glucose or gluconate to wild type and mutant cells growing on histidine. Glucose (and gluconate) appeared to exert a second effect at the transport level which interfered with inducer accumulation. Analysis of glucose- and gluconate-elicited catabolite repression of histidase in PA-1 and selected glucose mutants indicated that glucose and gluconate must enter the cells and be metabolized to cause repression; the effector of this repression was tentatively identified as 6-phosphogluconate.

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