# GENETIC, BIOCHEMICAL AND BIOENERGETIC STUDIES OF AN ADENYL CYCLASE DELETION MUTANT OF *ESCHERICHIA COLI* K-12

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## ABSTRACT

GENETIC, BIOCHEMICAL AND BIOENERGETIC STUDIES OF AN ADENYL CYCLASE DELETION MUTANT OF *ESCHERICHIA COLI* K-12

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An adenyl cyclase deletion mutant strain of *Escherichia coli* K-12 was studied to determine the role of cyclic adenosine monophosphate on growth and metabolism. The mutant strain was found to be sensitive to high environmental pH. The mutant made less cell mass per unit carbon provided and this deficiency increased at high environmental pHs, whereas the parent strain was resistant to environmental pH. Other *cya* deletion mutants exhibited similar properties. On this basis it was proposed that the *cya* mutants are unable to develop and/or maintain a normal electrical gradient across the cytoplasmic membrane. The pattern of uptake of $^{14}C$-proline by membrane vesicles at various external pHs in the presence and absence of valinomycin (an ionophore) and nigericin (a protonophore) supported this prediction. The hypothesis was further supported by the direct measurement of the two components of proton motive force, the electrical gradient and the proton gradient, in cells and membrane vesicles. Genetic studies show that *crp* gene product, the cAMP binding protein, is involved in developing resistance to the environmental pH.

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Dedicated to:

my mother,
Arish, Sabaa, Faiz
and
my husband.

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# INTRODUCTION

The work reported in this thesis deals with genetic, physiological and bioenergetic studies on a strain of Escherichia coli K-12 carrying a deletion in the cya gene.

The cya gene specifies the structure of the enzyme, adenyl cyclase, which catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP) and pyrophosphate (80, 83, 199):

$$ATP \xrightarrow{\text{adenyl cyclase}} cAMP + PP_i.$$

A deletion of this gene ( $^{\Delta}$cya) therefore renders the bacteria deficient in cAMP.

Cyclic AMP has been detected in a variety of living organisms, bacteria, animals and even plants (83, 153, 163). In humans and other mammals, it mediates the effect of a variety of hormones and biologically active agents (178). The work presented in this thesis is concerned with cAMP in bacteria.

Our understanding of the role of cAMP in bacteria has been advanced by the isolation of mutants deficient in it. The cya mutants show a wide variety of metabolic effects. The interrelationship between these is not obvious. One can however consider them in two groups—1) effects on transcription through which catabolite repression is mediated, and 2) effects on membrane structure and function.

Section 1. The Role Of cAMP In Catabolite Repression:

Many microbes, grown with glucose, are unable to metabolize various other sugars concurrently. This is because, during growth on glucose, the synthesis of enzymes to degrade other carbon sources (lactose, maltose, galactose and arabinose) is repressed. This phenomenon of repression was originally known as the "glucose-effect" [illegible]er studies showed that this repression is not specific to glucose. The presence of other rapidly metabolizable substrates in the growth medium exert similar but not identical repression. Based on this observation it was concluded that growth conditions that result in more catabolism than anabolism repress the synthesis of enzymes of other catabolic pathways (114, 204). The growth substrates that exert repression provide ATP at a faster rate.

This repression-renamed as catabolite repression- is mediated through cAMP (156). Cyclic AMP is needed to begin the transcription of catabolite repressed genes. However in glucose-grown cells, the concentration of cAMP is maintained at a very low level, such that these operons cannot be transcribed.

The evidence for this model is convincing. Glucose-grown cells have very low levels of cAMP (117). Addition of exogenous cAMP (along with the appropriate substrate) allows transcription of various genes (*lac*, *mal*, *gal*, *ara*, *deo* and *dsd*) even in the presence of glucose (65, 67, 75, 152, 157, 176, 206, 207).

The repression of the synthesis of these catabolite-sensitive enzymes has been ascribed to a phenomenon called "inducer exclusion", according to which the carbon source exerting repression prevents the entry of the inducer of these enzymes, thereby preventing the induction of their synthesis (114, 123, 204). However, it has been argued on the basis of physical and genetic studies that the phenomenon of inducer exclusion is only partially responsible for the catabolite repression (204). Cells in which lactose-catabolizing enzymes were induced by using an inducer analogue (isopropyl-β-thiogalactoside, IPTG) that can enter the cell without a transport system, were still sensitive to repression by glucose, and mutants constitutively producing maltose-catabolizing enzymes still retained some sensitivity to glucose. However, mutants constitutively producing galactose-catabolizing enzymes displayed no repression by glucose (81).

Ullmann et al. (202) have reported the presence of a factor, called catabolite modulator factor (CMF), in the water soluble extract of E. coli cells grown on glucose minimal medium. When added to a culture, this factor strongly inhibits the expression of genes sensitive to catabolite-repression (e.g., lac, gal, and tna) but it has no effect on those that are insensitive (e.g., genes for glucose-6-phosphate dehydrogenase and phosphoglucomutase). No correlation was found between the extent of catabolite repression and the amount of CMF present in the culture. However, some correlation was observed between the catabolite repression and the cell's capacity to degrade CMF (38). These workers characterized this factor as of

low molecular weight, acid-, base- and heat-stable and electrically neutral (38) but were unable to purify it.

The most conclusive evidence of the role of cAMP as the physiological effector in catabolite repression, however, was the isolation of mutants deficient in cAMP production (26, 145, 158, 186, 217). Such mutants were unable to use any of a number of sugars: lactose, maltose, arabinose, galactose, sorbitol and glycerol (123, 158). Fermentation of all sugars could be restored by exogenous cAMP.

This deficiency in the use of many carbohydrates provides an excellent screening method for *cya* mutants. A specific mutation in the *lac* operon would make a strain lactose-nonfermenting but still able to use maltose, arabinose, etc. However a *cya* mutant would be unable to use several carbohydrates (pleiotropic carbohydrate non-utilizing). This selection has been extensively used in the present work.

## Section 2. Discovery Of The Catabolite Repressor Protein (CRP):

Among the pleiotropic carbohydrate non-utilizing mutants isolated, many could be reversed by adding exogenous cAMP; however, many could not (155). Those not responsive to cAMP mapped in a different area of the *E. coli* linkage map. They are in a gene which specifies a cAMP binding protein, *crp*.

Mutants deficient in *crp* showed exactly the same phenotype as

those deficient in cya (47, 223); however this phenotype could not be reversed by cAMP. It seemed, therefore, that cAMP and CRP must act together to permit the synthesis of the various enzymes needed to degrade carbohydrates.

The CRP has been purified and studied in much detail (204). Its ability to interact with cAMP and DNA has been exploited for its purification. It is a basic protein consisting of two identical sub-units of molecular weight 22,500 each (43). Its amino acid sequence has been worked out (3, 30) and its three-dimensional structure has been established by x-ray diffraction studies (113). It appears to have two distinct domains— one, with amino terminus, interacts with cAMP, and the other, with carboxyl terminus, interacts with DNA. There are about 3500 molecules of CRP per cell in E. coli (63).

## Section 3. Mechanism Of Action Of cAMP And CRP In Catabolite Repression:

The mechanism of action of cAMP and CRP has been extensively studied, particularly with respect to the control of expression of the lactose, galactose and arabinose operons. That both cAMP and CRP are necessary for the initiation of transcription of these operons has been established through a number of genetic, biochemical and physiological experiments. In an in vitro purified system of transcription (consisting of lac DNA, RNA polymerase, nucleoside triphosphate and ATP), lac mRNA could not be synthesized from lac DNA

unless both cAMP and CRP were provided (36). Similar results were found when gal DNA (140, 150) or ara DNA (224) was used (155).

According to the currently accepted model, the cAMP/CRP complex acts at the promotor site of the catabolite-repressible operons, permitting RNA polymerase access for efficient transcription, and thereby allowing induction of enzyme synthesis. This model is supported by genetic and biochemical studies. In an in vitro transcription system using lac or gal DNA, the addition of nucleoside triphosphate and rifampin (a drug that inhibits the initiation of transcription by inactivating free RNA polymerase in the system) after the addition of cAMP, CRP and RNA polymerase did not interfere with the synthesis of lac or gal mRNA (29, 155). But these mRNAs were not synthesized when rifampin was added together with cAMP and CRP. This indicates that the cAMP/CRP complex affects the formation of the RNA-polymerase-promotor open complex. Physical conditions (e.g., high temperature) or chemicals that promote the formation of open complexes (e.g., glycerol, sulfoxide, ethylene glycol) increased the synthesis of gal or lac mRNA in an in vitro transcription system (31, 136, 137). These results strongly suggest that the cAMP/CRP complex is essential for RNA-polymerase to form an initiating complex at the promotor site.

CRP in the presence or absence of cAMP does not bind to RNA polymerase (141). However, in the presence of cAMP it does bind preferentially to a segment of lac and gal promotor regions (116). Thus, the promotor region, besides having a site for RNA polymerase,

appeared to have at least one other site for the cAMP/CRP complex. The interaction of the cAMP/CRP complex with the lac promotor region has been extensively studied using genetic and biochemical techniques, providing evidence for the concept that the promotor region has at least two binding sites (39, 56). Three distinct classes of mutations in the promotor region have been identified (155). One class of mutants has a low (2-6% of wild type) level of β-galactosidase. These mutants are resistant to glucose repression of β-galactosidase synthesis (159, 187) and the lac DNA from these mutants does not bind to CRP in an in vitro assay (116) suggesting that these mutations are in the cAMP/CRP binding site of the promotor region. The second class of mutants maps differently and has very low level of β-galactosidase (about 2%) in a $cya^+$ $crp^+$ background and this level is even lower in a $cya^-$ $crp^-$ background suggesting that these mutants are in the RNA polymerase binding site of the promotor region. The third class of mutants maps in the same region as do the mutants in the second class, but has a high level of β-galactosidase even in a $cya^-$ $crp^-$ background, indicating that the RNA polymerase binding site is altered in such a way that the enzyme can transcribe lac mRNA even in the absence of cAMP/CRP complex. The DNA base sequence of the lac promotor region has been identified (39) and these mutations have been described at the molecular level.

The reaction between cAMP and CRP is visualized as a reversible one, so that cAMP bound to CRP is in equilibrium with CRP and unbound cAMP (20). In this case, an increase in cAMP concentration would lead to an increase in the concentration of the cAMP/CRP complex (20).

The extent of catabolite repression then would be inversely

proportional to the intracellular concentration of cAMP/CRP complex (which itself is dependent on the intracellular cAMP concentration) and the interaction of cAMP/CRP complex with the promotor region of the catabolite sensitive operons. Since the base sequences of lac (39), ara (58, 191) and gal (198) promotors are not identical, it is suggested that the cAMP/CRP complex has different levels of affinities for different promotors (due to the base sequence differences). This would result in a sequential induction of operons, where operons with high affinity would be induced first with increasing concentration of the complex. Thus, the concentration of intracellular cAMP required to induce different operons is thought to differ. When different concentrations of cAMP were added to a culture of an E. coli cya mutant, β-galactosidase could be induced to 50% at an external concentration of $3.8 \times 10^{-4}$M, whereas arabinose isomerase induction required $8.6 \times 10^{-4}$M (111). This was also true in an in vitro transcription-translation system (165).

Normally then, cAMP binds to CRP and alters its conformation such that it can bind to DNA. That CRP undergoes a conformational change is shown by a number of physicochemical studies. The study of the pattern of the proteolytic digest (treatment with proteolytic enzymes) (44, 68, 102, 204), the reaction with cross-linking reagents (43, 149, 204), photoaffinity labelling (2) and the fluorescence studies (53), all provide evidence that CRP conformation changes on binding to cAMP. As previously stated, mutations in CRP may destroy its activity, presumably by interfering with its ability to bind cAMP

or to bind DNA. Another sort of mutation enables CRP to bind to DNA even in the absence of cAMP (5, 37, 125, 197). CRP from one such mutant has been demonstrated (through a protease digestion method) to exist in a conformation that is more like the CRP conformation when it is bound to cAMP (68).

The mutation which makes CRP independent of cAMP suppresses a cya mutation. Since cAMP is no longer needed for CRP function in such mutants, it clearly is irrelevant whether or not the strain makes it. Therefore a cya mutant carrying such a crp mutation (known as crp*) is able to use lactose, maltose and arabinose (5, 125, 197). In the present work, various new cya characteristics are shown to be suppressed by such crp* mutations.

## Section 4. Deficiencies Of The cya Mutants Are Not Limited To Carbohydrate Utilization: Slow growth rate with glucose:

The cAMP-deficient mutants differ from their parent strains in a number of other phenotypic characteristics. These differences are restored to normal when cAMP is provided, indicating that they are all due to cAMP deficiency.

One of the most puzzling characteristics is the slow growth of cya mutants in glucose minimal medium (50, 158). This has been explained in two ways. The first is based on the fact that cya mutants

transport substrates into the cell more slowly than cya$^+$ strains (50). From figures 8 and 9 of the paper by Ezzell and Dobrogosz (50), one can abstract that the wild type E. coli grown without cAMP transported a glucose analogue, $\alpha$- methylglucoside, at a rate of 3.5 n moles per mg cells per 30 sec, whereas the cya mutant grown without cAMP transported at 0.8 n moles per mg cells per 30 sec. The two strains grown with cAMP transported at the same rate and that rate. (7.0 n moles per mg cells per 30 sec) was higher than that of the parent grown without cAMP.

It is obvious that the presence of cAMP in the growth medium affected the activity of the glucose transport system. The question that is not clearly answered is whether the low transport rate in the mutant is, in fact, limiting for growth, i.e., if this decreased rate of transport of glucose is the reason for the slow growth rate. This cannot be answered from the data presented (50). However, Judewicz et al. have shown in E. coli Hfr 3000 that cells supplied with glucose and cAMP grow more slowly than those given only glucose (84), a contradiction to the above mentioned report.

A second explanation was based on the fact that the cya mutant's membrane showed low levels of the major heme constituents (cytochrome $b_1$ and o), and of flavine adenine dinucleotide, and had reduced NADH oxidase and energy-dependent transhydrogenase activities (27, 40). Because of these deficiencies in the respiratory chain components, it was implied that the cya mutant is not able to carry out efficient oxidative energy transduction (oxidative phosphorylation) and this

inefficiency results in a slow growth. From this, it was suggested that cAMP is involved in the regulation of the synthesis of a normal and fully functional aerobic respiratory chain.

Earlier studies showed that growing cells of E. coli B with cAMP and glucose increased the efficiency of ATP formation (76). That is, when cells had been growing on glucose and cAMP, they showed much higher P/O ratios (phosphate esterified per molecule of oxygen utilized) than when grown with glucose alone. Also consistent with this is the finding that exogenous cAMP added to E. coli K12 growing on glucose stimulated the synthesis of cytochromes ($b_1$ and o) (27).

The ability of the cya mutant to synthesize ATP was directly measured (40). Whether energized with a natural substrate (D-lactate) or with an artificial substrate (ascorbate-PMS), mutant cells synthesized less ATP than their parents (with D-lactate, the ATP level reached 3.5 and 1.0 mM within 30 sec in the wild type and mutant strains respectively; with ASC-PMS, it reached 4.25 and 3.5 mM in the wild type and mutant strains respectively). Since ATPase activity itself had been shown to be normal in cya mutants (40), the low rate of ATP synthesis was ascribed to respiratory chain deficiencies, resulting in insufficient energization of the membrane, and consequently lower ATP production.

Section 5. Other Defects Of The cya Mutant:

The cya mutants exhibit a number of other metabolic abnormalities, most of which can be ascribed in one way or another to alterations in membrane structure and function. As compared to a $cya^+$ strain, the mutant is more resistant to certain mutagens (108, 194, 204), antibiotics (7, 9, 20, 32, 108, 195, 204) and phages (4, 108), but more sensitive to high salt concentration (50) and to streptomycin (108). The outer-membrane protein composition (8, 20, 118, 135) and the regulation of synthesis of flagella (20, 42, 101, 217), pili (20, 71) and fimbriae (20, 46, 184, 204) are also altered. The mutant shows a normal phenotype in each of these characteristics if provided with cAMP exogenously.

The mechanism of action of cAMP in all these cases is likely to involve control of transcription, just as was the case for sugar utilization. This conclusion is based on the fact that crp mutants show similar characteristics. For instance, a crp mutant was salt sensitive, deficient in glucose transport and deficient in cytochrome $b_1$ and o, a phenotype very similar to that of the cya mutant (33). It also showed a decrease in total flavin content, decreased ability to make ATP and decreased transhydrogenase activity (40). It seems then that both cAMP and CRP are needed to establish these reactions.

The crp protein is thought to work exclusively as a DNA-binding protein involved in the control of transcription. If it is involved in the control of the growth rate on glucose, then establishment of the normal growth rate of E. coli must involve transcription at a cAMP/CRP

dependent promoter. The same is true of all the other functions, even though they are functions of glucose-grown cells and catabolite repression is not involved. A similar experimental logic is used in this thesis to show that pH-effects involve a function controlled by cAMP and CRP.

There are some reports that strongly suggest the role of cAMP as a negative effector in the synthesis of several E. coli proteins. This was first suggested by Prusiner et al. (171) who compared the levels of enzymes involved in the interconversion of glutamate and glutamine in the presence and absence of cAMP. The level of two of the enzymes was high in glucose grown cells and was decreased by cAMP. The finding that the cya and crp mutants have an increased level of these enzymes further confirmed the negative control of cAMP on these genes. Since the crp mutants have been known to have a high level of cAMP (synthesized by adenyl cyclase), the regulation of adenyl cyclase may also be negatively controlled by cAMP/CRP (170, 209). A study involving two dimensional polyacrylamide gel electrophoresis of glucose grown cells from the wild type and the cya and crp mutant strains has shown that a large number of proteins are negatively controlled by the cAMP/CRP complex (118). One of these negatively controlled proteins has been identified as the outer membrane protein III (synthsized by gene ompA). Since the CRP is necessary for mediating the repressive effect of cAMP it seems likely that the negative control is exerted at the level of transcription, as is the positive control. Whether this control is direct or indirect

(through the transcription of a regulatory protein), is still to be discovered.

Cyclic AMP has been suggested to be involved in relieving the polarity in the polycystronic transcriptional units (operons). In an operon the individual genes are expressed co-ordinately. However, the expression of promotor-proximal genes is higher than the promotor-distal genes. In the case of lac and gal operons, this polarity of transcription has been found to be relieved by cAMP (203, 204). This antipolar role of cAMP and the underlying mechanism is not yet well understood.

Recently, it has been proposed that cAMP plays a role in transcription termination (60, 203, 204). This proposition is based on the comparative study of the effect of a temperature sensitive rho mutation in the wild type and in the cya/crp mutant strains. In the presence of a non-functional rho gene product (the transcription terminator protein), the transcription once started proceeds almost without termination, leading to an unbalanced expression of the genome. Consequently, the growth stops. Mutations in the cya or crp genes are able to suppress the effect of rho mutation, indicating the involvement of the cAMP/CRP complex in transcription termination. From these pleiotropic carbohydrate-negative (cya/crp) rho mutants, pleiotropic carbohydrate-positive pseudorevertants have been isolated that map in rpo gene. The rpo gene transcribes the sigma subunit of RNA polymerase. In a recent report, it has been proposed that the rpo gene takes part in the regulation of cAMP synthesis, since a

temperature sensitive rpoD800 mutant contains a decreased level of cAMP in the culture (60).

It has been suggested that the cAMP/CRP complex plays a role in the biosynthesis of branched chain amino acids (34, 35). The relA mutants, that exhibit a relaxed control of the synthesis of stable RNAs on amino acid starvation, have been found to be sensitive to serine, methionine and glycine, SMG (35). The fact that this sensitivity was relieved by the addition of isoleucine or by mutations in the cya or crp genes, provided a basis for the above mentioned role of cAMP/CRP complex. A further evidence was provided by the finding that the crp* mutants excrete $\alpha$-ketobutyrate (a precursor of isoleucine) when grown on glucose. Moreover, the SMG-resistant mutants have been found to be sensitive to $\alpha$-ketobutyrate and its analogues, and to have altered cAMP accumulation (34). However, it is not yet known how and at what step the cAMP/CRP complex plays its role in the biosynthesis of branched chain amino acids.

## Section 6. Cloning of Adenyl Cyclase Gene (cya):

Recently, microbiologists have started applying tools and techniques of genetic engineering in order to comprehend the complex and yet not well understood role of cAMP in metabolism. The adenyl cyclase gene from E. coli (180) and Salmonella typhimurium has been cloned into the plasmid pBR322 (208). In E. coli the characterization

of regulatory regions by operon fusion (*lac* Z and $tet^R$) and the study of gene product, indicate the existence of three promotors. Two of these initiate upstream transcription of *cya*, synthesize a 95,000 Da polypeptide and are insensitive to cAMP (180). The third one is also in the same control region but transcribes in the opposite direction. The sequencing of these control regions is underway (204).

Wang *et al.* (208) cloned restriction fragments containing *cya* gene from *S. typhimurium*. The size of the DNA cloned varied as judged by the size (from 78,000 to 81,000 Da) of the polypeptides made. A plasmid carrying a partly deleted *cya* gene, synthesizing a polypeptide of 45,000 Da, was able to complement a $\Delta$*cya* strain. A similar study of even larger deletions of the carboxyl-terminal end of the *cya* gene did not affect the enzyme activity (109). Deletion mutations producing polypeptides as small as 46,000 Da did not have a significant effect on the enzyme activity. Larger deletions giving polypeptides of up to 33,000 Da had no detectable enzyme activity but were still able to complement a *cya* mutant. These results suggest that the active site of adenyl cyclase may be contained in a very small domain of the enzyme molecule and that the rest of the molecule may have other functions (109).

Section 7. Control Of cAMP Synthesis And Excretion:

As described in the preceding sections, repression of the synthesis of the various catabolite-sensitive enzymes appears to be mediated by cAMP. If cAMP is the mediator, then the fact that the induced level of the catabolite-sensitive enzyme is different in cells grown with different carbon sources, should imply that the cAMP concentration of the cell is also different. That is, there should be a correlation between the intracellular level of cAMP and the rate of synthesis of catabolite repressible enzymes. Hence the extent of catabolite repression should depend primarily on the concentration of cAMP in the cell.

This prediction could be tested most directly by the measurement of the internal cAMP concentration of cells grown with various carbon sources. However the determination of intracellular cAMP is difficult in bacteria, and subject to many errors. This is because cAMP is present in a very low concentration in bacterial cultures, and most (roughly 98%) of it is excreted into the medium (20, 155, 176, 204). A slight contamination of cells by extracellular liquid will then greatly alter the values obtained. Moreover, the intracellular concentration changes rapidly when the cells are subjected to physiological stress, as in centrifuging or filtering.

The difficulty of this determination is reflected in the considerable variation in published values for cellular cAMP concentration. For glucose-grown E. coli, cAMP concentrations of 0.4 (48), 1.5 (152, 155), 9 (209) and 12 μM (28) have been reported; the

same studies reported that glycerol-grown cells had concentrations of 1.5, 5, 19 and 43 μM, respectively. These studies all depended on antibody-binding to measure cAMP; however, they varied in the way the sample of cAMP was obtained, i.e. in the method of filtration, the extent of washing and the method of extracting cAMP. It is not obvious from these studies what the absolute value of cAMP may be. Still, it is noteworthy that in all cases, glycerol-grown cells had considerably more cAMP than glucose-grown cells.

That glucose-grown and glycerol-grown cells have different cAMP concentrations is also indicated by studies that show that the addition of glucose to glycerol-grown cells causes a rapid decrease in cAMP concentration. Shifts from 5 to 0.5 μM (152), from 1.5 to 0.2 μM (48), from 14 to 2 μM (209), and from 33 to 10 μM (28) in 5, 20, 20 and 5 minutes, respectively, have been reported.

Investigators have tried to avoid the problem that cAMP concentration varies rapidly with changes in growth conditions by making determinations on cells grown in continuous culture. In one study with glucose-grown (repressed) cells, using various dilution rates, values of cAMP from 0.27 to 0.32 μM were reported (215). A similar study reported values of 0.29 to 1.0 μM (122). Glucose-limited cells (derepressed) and succinate-grown (derepressed) cells gave values of 4.4 to 5.5 and 0.8 to 2.8 μM, respectively, in the former study, again suggesting that glucose-grown cells have less cAMP than less catabolite-repressed cells.

In a more recent study using continuous cultures, the intracellular cAMP concentration turned out to be almost the same in both the glucose-limited (catabolite derepressed) and the phosphate-limited (catabolite repressed) cultures, around 1 μM, whereas the external concentration varied greatly: between 0.4 and 1.0 μM in the former, and around 0.05 μM in the latter (122). Recently, a new technique has been developed in which the removal of cells from the culture is avoided and the cAMP-phosphodiesterase is used to degrade the extracellular cAMP (82). Using this technique in glucose grown culture the intracellular cAMP concentration was found to be two times higher than that in glycerol grown cells.

A more detailed study of the correlation between the extent of catabolite repression exerted by different carbon sources on cAMP content was made by Epstein et al. (48). They measured intracellular cAMP and β-galactosidase activity in cells grown on 13 different carbon sources, in each case with isopropyl-β-D-thiogalactoside (IPTG) as the inducer of β-galactosidase. The good correlation between β-galactosidase activity in the cell and cAMP concentration indicates that whatever the absolute value of cAMP may be, its relative value varies as a function of the substrate used.

The intracellular cAMP concentration may be affected by various factors. The rate of cAMP synthesis may vary, due either to a change in the rate of synthesis (transcription) of adenyl cyclase, or to the variation in its activity, possibly caused by the presence of inhibitors and activators. Cyclic AMP concentration may also be directly affected by alterations in the activity of the enzyme

that degrades cAMP, cAMP phosphodiesterase or by alterations in the rate of its excretion into the medium, or of reimport from the medium. Excretion seems to be energy dependent (57, 182); uptake does not (182).

Makman and Sutherland showed that glucose-grown cells when starved for carbon increased their cAMP content within 15 minutes (117). Similarly, when glucose was re-added, the cAMP concentration decreased. Since both changes were also seen when chloramphenicol was present, they cannot be due to protein (adenyl cyclase) synthesis. Moreover the changes in cAMP concentration in these and other experiments were much more rapid than would be expected of changes due to altered rates of the synthesis of adenyl cyclase. Control of cAMP concentration is therefore more likely to be due to an effect on the activity of adenyl cyclase rather than on its synthesis.
These authors suggested that the addition of glucose stimulated the excretion of cAMP and suppressed its formation in the cell.

Adenyl cyclase activity in E. coli B grown with glucose was inhibited by glucose, as judged by assays in whole cells and in toluene-treated cells (72, 161). However, in extracts made with the French press, adenyl cyclase activity was not sensitive to glucose (72). These in vivo assays depend upon the conversion of radioactive ATP (substrate of the enzyme) to cAMP. Since the cAMP is radioactive, it can be detected more readily with binding proteins. These determinations are therefore likely to be reliable. The enzyme activity assayed by this technique was high enough to account for the

quick changes in the intracellular concentration of cAMP (72, 163).

Adenyl cyclase activity is not inhibited by glucose-6-phosphate (160), suggesting that inhibition is by glucose itself. This conclusion is supported by the fact that the activity is inhibited by a nonmetabolizable analogue of glucose, β-methylglucoside (155, 183).

The fact that glucose inhibits adenyl cyclase in whole cell preparations, but not in extracts, suggests that its effect is not directly on the adenyl cyclase activity. The suggestion has been made that it is the transport of glucose into the cell that inhibits adenyl cyclase (162), the underlying argument being that the adenyl cyclase activity in vivo was not only inhibited by glucose but also by some other sugars: sugars that were used for growth and whose transport systems were induced. The activity was inhibited by sugars transported by a variety of mechanisms — glucose and mannitol transported by the phosphoenol pyruvate (PEP) dependent transport system, lactose transported with proton, and glycerol transported by facilitated diffusion (41, 162, 169, 183).

Glucose enters the cell through the PEP-dependent transport system. The phosphoryl group is transferred from PEP to glucose via a cascade of phosphorylating proteins (204). This phosphorylating system (Figure 1) is also used for the transport of other sugars, e.g., fructose, mannose, sorbitol, mannitol, etc. Two of these proteins, Enzyme I (EI) and a heat stable protein (HPr), are common to all sugars transported by this system. These are coded by genes ptsI

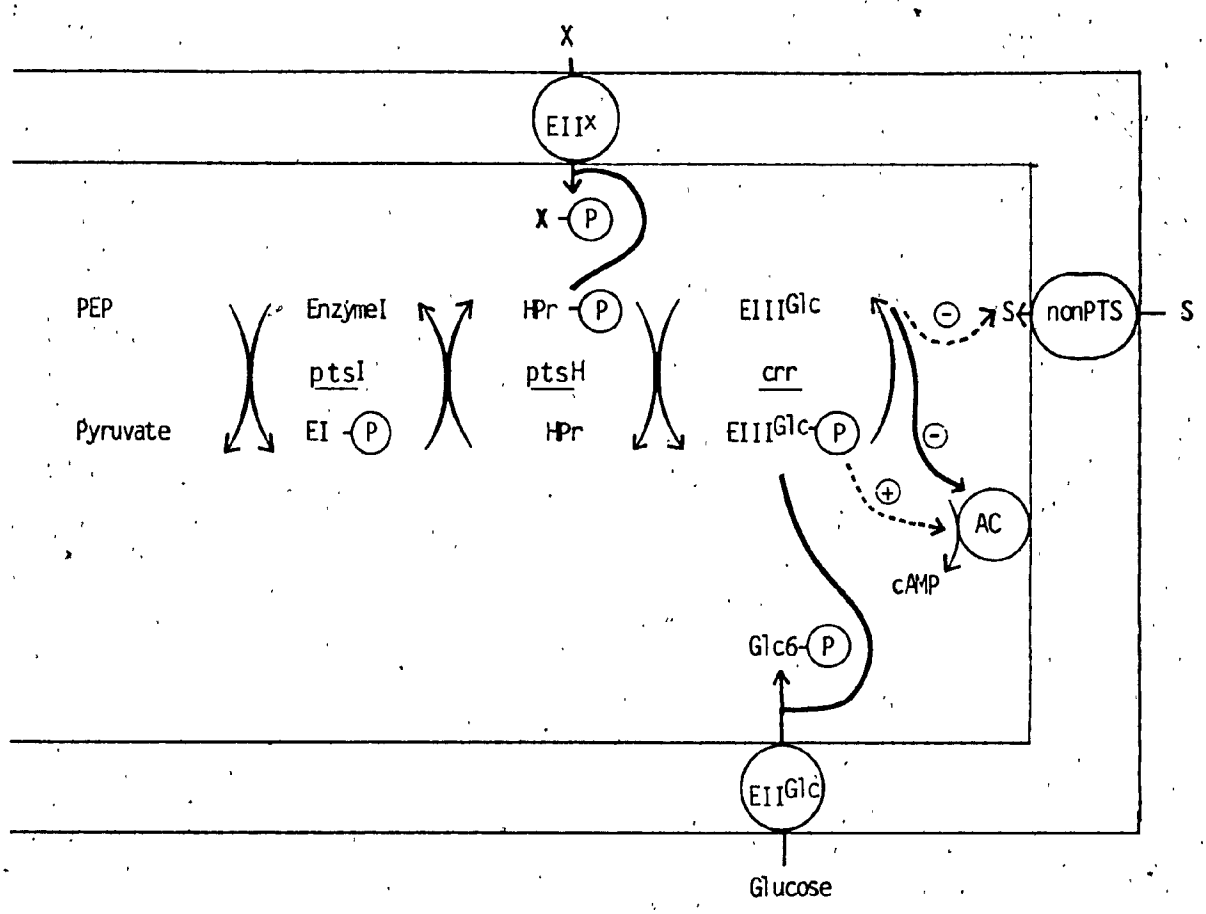



Figure 1: A model for the phosphoenol pyruvate dependent phosphotransferase system of regulation of carbohydrate transport and adenyl cyclase. (taken from 204).

⊖ Inhibition
⊕ Activation
(P) Phosphoryl group
AC Adenyl cyclase
S nonPTS substrate
X PTS substrate (except glucose)
Glc Glucose
Glc6-(P) Glucose-6-phosphate
PEP Phosphenol pyruvate

and pstH and are cytoplasmic. Another protein, Enzyme II (EII), is membrane bound and is specific for each sugar. Besides these three proteins, there is one more protein coded by crr gene (Enzyme $III^{glc}$) that is involved in the transport of glucose. According to this model, PEP phosphorylates EI, which in turn phosphorylates the HPr. The phosphorylated HPr transfers the phosphoryl group to the sugar specific EII and EIII. These enzymes transport sugar into the cell in their phosphorylated form. It was also suggested that the phosphotransferase system (PTS), in the absence of the sugar, phosphorylates the enzyme (adenyl cyclase). Adenyl cyclase was thus presumed to be active only in a phosphorylated form. When glucose is transported, the component of the PTS is used to phosphorylate glucose, and adenyl cyclase reverts to an inactive, nonphosphorylated state for as long as there is enough glucose to drain off phosphate.

It was originally suggested that enzyme I of the PTS was the phosphorylating agent (154). However a mutant of E. coli which carries a deletion in enzyme I has a normal level of cAMP (216). In Salmonella, Saier suggests that the crr gene product, enzyme III, may be involved (181, 183).

Peterkofsky and Gazdar showed that adenyl cyclase is also inhibited by the transport of lactose, and of the nonmetabolizable analogues, IPTG and thiomethyl galactoside (TMG) (164). These compounds are not transported by the PTS. Lactose is transported by proton symport (70), thus utilizing the energy from proton motive force (PMF) across the cytoplasmic membrane. This led them to suggest

that adenyl cyclase activity varies with the proton motive force. The fact that the addition of protonophores decreased adenyl cyclase activity led them to deduce that adenyl cyclase function is inhibited as the PMF is decreased. Given the fact that during exponential growth, the cell maintains a more or less constant PMF, it becomes difficult to understand that lactose transport would decrease the PMF. It seems unlikely, then, that the relation between PMF and adenyl cyclase can be as direct as these authors suggest.

There is some genetic and physiological evidence that the catabolite repressor protein (CRP) also affects the rate of adenyl cyclase synthesis (19). For these studies, cells were grown in a variety of conditions, resuspended without any carbon source (to avoid the inhibition of the enzyme activity by its transport), and the amount of cAMP produced on incubation was determined. The rate of cAMP production is then taken as a measure of the amount of adenyl cyclase present in the cells. This protocol is referred to as the "aeration assay" for adenyl cyclase activity.

In these experimental conditions, glucose-grown wild type cells made approximately twenty times more cAMP than either glycerol- or succinate-grown cells. This suggested that glucose-grown cells have more enzyme, though its activity is inhibited, i.e. the transcription of adenyl cyclase is repressed when cells grow on succinate or glycerol and derepressed when cells grow on glucose. Thus, when glucose is exhausted from the medium, it seems possible that the cells could respond rapidly with an increase in cAMP.

The involvement of CRP was indicated by the fact that a crp mutant made a high level of cAMP in the aeration assay just described. This was true for the crp mutant, no matter which substrate was used — glucose, glycerol or fructose. In a mutant, in which CRP was altered so as to function without need for cAMP as a cofactor, cAMP production was low, and was also insensitive to the substrate. This suggests that adenyl cyclase activity responds to alterations in the crp protein.

This involvement of CRP was also suggested by similar experiments of another group of investigators, using the same assay for cAMP production, and also using an assay based on the formation of radioactive cAMP (115). They also grew cells with glucose-6-phosphate, and showed that cAMP production in the aeration assay was inhibited by glucose-6-phosphate. This is different from the earlier report that adenyl cyclase is not inhibited by glucose-6-phosphate. These authors took this to support the idea that adenyl cyclase is controlled by CRP, rather than by glucose transport system. However, it is not clear that glucose-6-phosphate is not hydrolyzed to glucose in their experiments.

The level of cAMP might also be controlled by variations in phosphodiesterase activity. E. coli K-12 does have phosphodiesterase activity (139). It is unlikely that the cAMP concentration is governed by changes in the rate of synthesis of this enzyme, since glucose- and glycerol-grown cells showed the same activity (19, 139). A mutant deficient in phosphodiesterase (cpd) did show an increase in

cAMP concentration, but a smaller increase than would be expected if this enzyme were a major factor in regulating its concentration (19). Thus, it is also likely that this activity is involved to some extent in regulating the level of cAMP, because the carbon source used affected the cAMP concentration in both the mutant and wild type strains. However, the Km for this enzyme is two-fold higher than the in vivo concentration of cAMP, which perhaps makes it unlikely that it acts sufficiently quickly to regulate the cellular cAMP concentration.

Another factor in the regulation of cellular cAMP concentration may be the excretion of preformed cAMP from the cell. When glucose was added to cultures growing on glycerol, cAMP was rapidly excreted into the medium (209). In a similar study quoted earlier, Epstein et al.(48) showed that extracellular cAMP, as well as intracellular cAMP, varied with the substrate used to support growth and that this variation paralleled changes in steady-state β-galactosidase concentration. Thus, it seemed that the cell excreted cAMP at rates proportional to the cAMP concentration in the cell (48). Roughly 99% of cAMP was excreted under all conditions tested (48, 209).

Excretion of cAMP was shown to require energy. Inside-out vesicles did not take up cAMP from the medium unless they were energized (57, 182). If cAMP is synthesized at a fixed rate, which depends perhaps on the carbon source, and if excretion requires energy, then when cells exhaust their energy supply, thus blocking the excretion, they would continue to synthesize cAMP. The small amounts needed to increase cellular concentration of cAMP would not require a

significant energy input, and the cells would then accumulate intracellular cAMP, allowing adaptation to new substrates.

Section 8. Bioenergetics And pH-Sensitivity:

The experiments in this thesis demonstrate a new characteristic of the cya mutant— its sensitivity to high environmental pH. Whereas the normal E. coli grows at much the same rate from pH 6 to 8, the cya mutant grows slowly at pH 6, and increasingly slowly as the pH is increased. This appears to be due to its problems in energization of the membrane. For this reason, a preliminary discussion on membrane energization and pH-sensitivity is presented here.

The energy available to an organism from its environment is converted into energy forms that can be used to drive its cellular reactions. A large portion of the metabolic energy is conserved and transduced specifically at membranes of a particular type. The cytoplasmic membrane in procaryotic cells, the inner membrane of mitochondria in eukaryotic cells and the thylakoid membranes of chloroplasts in plant cells serve as the "energy transducing" membranes. The general pathway by which energy is harnessed for biological purposes is the same in bacteria, mitochondria and chloroplast. Mitchell proposed in 1966 that this pathway operates by a process known as chemiosmosis. Since then a large amount of experimental evidence in favour of this theory has made it the most

widely accepted theory of energy conservation (70, 128, 134, 138, 188). According to this theory, the process of energy transduction begins with the transfer of high-energy electrons that have either been trapped in electron-rich (reduced) food materials or excited by sunlight, along a chain of proteins known as electron-transport proteins embedded in the membrane. During their descent down the chain, those high-energy electrons fall successively to lower energy levels. The energy released by this "down-hill" transfer of electrons results in a proton-translocation across the membrane due to the spatial organization of the components of the electron-transport chain. The electron-transport proteins are vectorially arranged, spanning the membrane. These consist of alternating sequences of hydrogen carriers and electron carriers. Because of the arrangement of the carriers through the membrane, oxidation of electron-rich substrates occurs on the inside of the membrane, with the transfer of hydrogen to the neighbouring hydrogen-carrier, also on the inside of the membrane. This carrier transfers electrons to an adjacent electron-carrier, and ejects the remaining proton across the membrane. Since the membrane is impermeable to protons, as a consequence of proton extrusion, there develops, across the membrane, an electrical gradient, $\Delta\psi$ , (inside negative due to the extrusion of positive charged protons) and a pH difference, $\Delta$ pH, (inside alkaline due to the loss of protons). These gradients exert a force on the protons, tending to pull them back across the membrane. This inwardly directed force on the protons is called the proton motive force (PMF)

or the electrochemical proton gradient ($\Delta p$). The energy stored in this gradient can in turn be harnessed by other proteins in the membrane. In mitochondria and chloroplasts the PMF is used to make ATP through another membrane bound protein - the ATP synthetase (usually known as the ATPase). In bacteria the PMF itself is as important a source of directly usable energy as is ATP. It is used to make ATP, to carry out active transport of solutes and for motility.

Mitchell's chemiosmotic theory had four basic requirements each of which had been verified through a number of experiments (59, 70, 133, 134, 138, 188). The first requirement, that the electron transport chains translocate protons, was verified by showing that the addition of a small pulse of oxygen to an anaerobic suspension of mitochondria results in the acidification of the medium (129, 131). In inverted submitochondrial particles the medium became basic. The second requirement, that the ATPase functions as a reversible proton-translocating pump, was verified by showing that the addition of ATP into an anaerobic suspension of mitochondria results in the acidification of the medium (132). The third requirement, that the energy-transducing membranes are proton-impermeable, was proved by measuring the rate of decay of an HCl-induced pH gradient across the mitochondrial membrane (130). The fourth requirement, that the energy-transducing membrane is equipped with a set of specific carrier proteins to permit the entry and exit of essential metabolites, was verified by showing that the decay of $\Delta$pH, developed by giving a pulse of oxygen to an anaerobic suspension of mitochondria, is

accelerated by the presence of succinate, Pi, malonate and $Na^+$ (130).

All these requirements of Mitchell's theory have been verified in bacterial, mitochondrial and thylakoid membranes, in artificial lipid membrane, in sub-mitochondrial particles, in membrane vesicles, in photosynthetic bacteria and in reconstituted membrane systems.

The two components of the PMF, $\Delta\psi$ and $\Delta$pH are interconvertible, and are usually expressed in electrical units, millivolts (mV). The PMF is expressed as the sum of the two components:

$$PMF(mV) = \Delta\psi(mV) - Z\ \Delta pH\ (pHin - pHout)$$

where $Z = 2.303RT/nF$ (R = Gas constant, T = Absolute Temperature, F = Faraday's constant, n = 1)

$$Z = 59 \text{ at } 25°C.$$

Therefore, $$PMF(mV) = \Delta\psi(mV) - 59\ \Delta pH.$$

There is an increasing controversy over the extent to which the proton translocated across the membrane goes into the bulk aqueous phase. According to Mitchell's chemiosmotic theory, the protons flow (circuit) between the electron-transport chains (or any proton motive source) and ATPase (or any proton sink), through the bulk aqueous phase (134, 138). Thus, according to this model, the membrane serves as a passive barrier for ions and solutes. The other view is that the protons do not enter the bulk phase, but remain attached to the membrane surface and form local proton circuits between the source of proton motive force and proton-sink (96, 97). These circuits create the so-called "protoneural network" in the membrane/solution

interphase. Arguments in support of this model are based on the findings that a single molecule of the proton-translocator, gramicidin, results in the total uncoupling of a chloroplast (mentioned in 138), and that in E. coli a single molecule of colicin kills an entire cell by rapid loss of both the intracellular $K^+$ and the electrical gradient (97). This "single-hit" killing action of gramicidin and colicins can not be well explained by the delocalized proton circuit theory of Mitchell, because it is hard to understand how a single gramicidin or colicin channel can bring about a rapid loss of the bulk phase membrane potential. However, a more plausible explanation can be given by following the view of local proton circuit. A single adsorbed molecule could then be considered as affecting the protoneural network, hence causing a rapid loss of membrane potential. If one maintains this view, then the bulk phase electrochemical proton gradient across the membrane should be considered as a consequence of secondary ion movements in response to the primary proton translocation. And then the functionality of this gradient in synthesizing ATP will be questionable. The fact that in halophilic bacteria the proton pumps and the ATPase complexes are concentrated in two separate regions of the membrane favours the bulk proton theory and goes against the local proton theory (45, 138).

The bulk phase PMF has been measured in a number of bacterial cells and vesicles by separately measuring its two components, $\Delta\psi$ and $\Delta pH$. The electrical gradient ($\Delta\psi$) is usually determined by measuring the concentration gradient at equilibrium, of an ion that is

transported through the membrane in its charged form or by measuring the equilibrium distribution of spectroscopic or radioactive indicators of $\Delta\psi$ (51, 52, 138, 146). The pH gradient ($\Delta$pH) is determined from the equilibrium distribution of weak acids or bases that are transported in their electroneutral form. The selection of an appropriate probe is very crucial in these measurements (52, 138, 146). This indicator should not be transported through more than one mechanism, and should not be metabolized. It should not be bound significantly or its binding should be measurable. It should distribute itself readily and, most importantly, it should be of the correct charge. It should be a cation for measuring an inside negative $\Delta\psi$ and an anion for an inside positive $\Delta\psi$. For measuring an inside alkaline $\Delta$pH it should be a weak acid and for an inside acid $\Delta$pH it should be a weak base.

In E. coli, the PMF has been reported as 207 and 140 mV at pH 6 and 8, respectively (218). Corresponding values for Streptococcus lactis, were 143 and 133 mV at pH 5.1 and 6.8 respectively (92). However, the $\Delta$pH in E. coli changed from 105 mV at pH 6 to -11 mV at pH 8, while the $\Delta\psi$ went from 102 to 152 mV (218). Similar changes were seen in S. lactis, where the $\Delta$pH decreased from 60 to 25 mV as the $\Delta\psi$ increased from 83 to 108 mV at pH 5.1 and 6.8, respectively (92). Thus the magnitude of the PMF varies only slightly with the external pH ($pH_o$) but the magnitude of its two components is greatly influenced by it.

The active transport of a number of sugars and amino acids across

the membrane is energized by the PMF (16, 54, 55, 70, 85, 86, 88, 90, 94, 100, 121, 173, 201, 218). In E. coli cells and membrane vesicles, the steady state accumulation of a number of transport substrates has been found to be correlated with the magnitude of PMF which was manipulated by using varying concentrations of the uncoupler, FCCP (218) and the ionophores, valinomycin and nigericin (173, 201). Very recently, a mutant of Streptococcus pneumoniae that has a low value of $\Delta\psi$ (and therefore a low PMF), has been shown to have a deficiency in the rate of $\Delta\psi$-dependent transport of some amino acids (201). These results indicate that there is a correlation between PMF and the active transport of some amino acids. In the same report it has been shown that the reduction of $\Delta\psi$ by $TMPP^+$, also decreases the rate of amino acid uptake. The proton motive force, in E. coli, has been calculated by using the steady state accumulation of lactose (94, 95, 218) in a lac operon constitutive, β-galactosidase negative mutant.

The steady state level of accumulation of neutral solutes has been found to be relatively unaffected by the external pH, as is the PMF (94, 173, 218). This indicates that the functionality of the PMF in active transport of these solutes remains constant, even though its components change.

According to Mitchell, the energy metabolism of a bacterial cell functions by circulation of protons between the cytoplasm and the medium (134). The cell must extrude protons in order to energize its membrane, which in turn drives the uptake of solute, a function vital

for the cell. In aerobes, the extrusion of protons is a consequence of the manner in which the electron transport chain is situated in the membrane. In anaerobes the extrusion of protons is due to the ATPase activity and in halobacteria it is caused by the light-driven proton pumps. In E. coli which is a facultative anaerobe (i.e., it can grow under both aerobic and anaerobic conditions), the membrane is energized by electron transport under aerobic conditions and also under anaerobic conditions, if an electron acceptor is provided (e.g., nitrate or fumarate). Under anaerobic growth in the absence of any electron acceptor, membrane energization is carried through the hydrolysis of ATP made available by substrate level phosphorylation. Thus, a mutant lacking ATPase activity is unable to grow anaerobically on glucose (64, 69, 70, 138). Strict anaerobes and aerobes can not survive without ATPase because the former have no alternative way to energize the membrane and the latter have no alternate way to make ATP (having no substrate level phosphorylation). The extrusion of protons thus becomes a function vital to life. However, the cell must also maintain a constant internal pH ($pH_i$), since its internal machinery is designed to function at a particular pH (as indicated by the pH optima of various cell activities) (18, 147). It is maintained between 6.4 and 6.9, 7.6 and 7.8, and 9 and 9.5 in acidophilic (78, 79, 104, 107, 147), neutrophilic (18, 147, 218) and alcalophilic (107, 120, 147) bacteria, respectively.

In E. coli, a neutrophile, the $pH_i$ is kept between 7.6 and 7.8. Extrusion of protons tends to make the cell interior more alkaline,

requiring it to acidify its cytoplasm. The cell has no problem in acidifying the cytoplasm if the external pH is less than 7.8 because the proton concentration gradient is directed inwards. If the external pH is 7.8, and the internal pH is kept constant, there can be no concentration gradient of protons ( $\Delta$pH) across the membrane. In this case, the extrusion of protons would tend to make the cell interior more alkaline than 7.8. However, excessive alkalinization cannot be tolerated. The cell circumvents this by setting some cation/proton exchange reactions in order to compensate for the subsequent decrease in $\Delta$pH with an increase in $\Delta\psi$. This allows it to keep the internal pH constant (7.6-7.8 over an external range of pH 5 to 8), and still maintain a constant PMF.

This complex system of pH-homeostasis of cytoplasm is thought to function in the following way. The respiratory chain proteins continuously pump out protons at a fairly constant rate over the entire external pH range at which the cell functions — i.e. pH 6-8. This is believed to be the case because the rate of oxygen uptake does not change significantly with pH (89, 103, 146, 174), and the oxygen uptake rate is a function of the activity of the electron transport chain. The extrusion of protons then causes the cell to become increasingly alkaline. If this process continued without compensation, the cell would quickly drive itself beyond the internal pH levels it can tolerate. Protons must therefore reenter the cell to keep the $pH_i$ constant. The current hypothesis is that the process of membrane energization starts with the extrusion of relatively few protons

across the membrane developing a small $\Delta pH$ but a large $\Delta\psi$, since the development of $\Delta\psi$ needs movement of relatively few charges. Then, in response to this $\Delta\psi$, the protons move into the cell through the cation/proton exchangers (antiports) and reduce the $\Delta\psi$. In response to this decrease in $\Delta\psi$, more protons are extruded and a higher $\Delta pH$ is developed (103, 138, 189, 190). Eventually an alkaline $pH_i$ is reached at acidic external pHs. To maintain this $pH_i$, the cell has relatively lesser difficulty when the external pH is low, because in this case, the internal milieu is more alkaline than the external pH and the cell can maintain a high $\Delta pH$. Protons are extruded and are returned to the cell through the ATPase and cation/proton antiporters, in symport with whatever substrates that are being transported and perhaps through some leak.

As the external pH increases, however, the magnitude of the $\Delta pH$ must decrease (if the internal pH is to be kept constant, as it is). Since the rate of proton extrusion is nearly constant at all pHs, there must be an increased rate of proton return in such a way that the $\Delta\psi$ is increased instead of being dissipated. Mitchell in 1966 postulated that this is done by various ion exchangers (103, 138, 146, 189, 190). Since then, three distinct antiport systems have been described in E. coli, all extruding cations from the cell in exchange for protons. These are the calcium proton antiport system (CHA) which transports divalent cations (23, 24), the sodium proton antiport system (NHA) which transports sodium and lithium ions (14, 23, 185, 210, 219, 220, 221, 222) and the potassium proton antiport system

(KHA) which transports potassium and other monovalent cations (1, 25, 103, 166).

The sodium/proton antiport has been reported in alcalophilic bacteria, Bacillus firmus (106), B. alcalophilus (105) and Exiguobacterium aurenticum (124 ), and in a strictly anaerobic sulpher reducing bacterium, Desulfovibrio vulgaris (205). The potassium/proton antiport has been reported in a marine bacterium Vibrio alganolyticus (200). The $Na^+$, $K^+$ and $Ca^{++}$ transporters have been reported in some streptococci (74, 151).

What happens in a neutrophilic bacteria like E. coli at high pH, say pH 8.2? As mentioned before, the oxygen uptake rate does not change significantly. Now the $\Delta$pH is in the reverse direction, i.e. the external pH is more alkaline than the internal pH limit. At steady state, if the net import of protons (by the antiporters, by the ATPase and with the solute import) still equals the net proton extrusion (by the primary proton pumps), the internal pH will increase and will equal the external pH. The overall proton influx must then increase further to maintain an internal pH which is more acidic than the external pH. For example, if n-1 protons are pumped out through respiratory chain proteins, and n protons are imported back into the cell with extrusion of n-1 cations through the antiporters, then the result will be a net movement of one proton (n-1 extruded and n imported) into the cell, causing the cell, to be more acidic, and one net positive charge out of the cell so that the electrical gradient is still maintained. This means that when the external pH is greater

than 7.8, the antiport activity must become electrogenic. This may be achieved, when the pH is above 7.8, by increasing the activity of cation/proton antiporters or by the activation of a new porter(s) or by a change in the stoichiometry of the proton taken in per cation extruded by the antiporter(s) from one (electroneutral) to more than one (electrogenic). In fact, potassium/proton and calcium/proton antiporters showed a pH-optimum of 8 in everted membrane vesicles (24, 25). But the validity of these measurements has been questioned, since the $pH_o$ in these vesicles is actually the $pH_i$ of the cell (174). Another study on everted membrane vesicles indicated that the calcium/proton antiporter operates by an electrogenic mechanism (24). Similarly, the sodium/proton antiporter has been shown to be electrogenic in right-side out membrane vesicles (185). The sodium/proton antiporter in cells from a strict anaerobic sulfer-reducing bacterium, Desulfovibrio vulgaris, has been shown to be electrogenic (205). Although not quite conceivable and not yet shown, the sodium/proton antiporter has been proposed to change its stoichiometry ($Na^+_{out}/H^+_{in}$) from electroneutral at external pHs below 7.8 to electrogenic at pHs above 7.8 (147).

One would expect that cells defective in cation/proton antiport activity might have a problem in maintaining $pH_i$ at external pHs of 7.8 and above. This is because if they lack the cation/proton antiport activity, they could not compensate sufficiently for proton extrusion and the interior of the cell would become alkaline. Mutants lacking either sodium/proton antiport activity (61, 62, 105, 146, 219,

220, 221) or potassium/ proton antiport activity (166) have been isolated. Both were found to be sensitive to alkaline pHs because of their failure to establish pH-homeostasis of cytoplasm although they were able to grow normally at acidic and neutral pHs (18, 103, 147). This implies that the cell needs both of these antiporters functioning for returning protons at a sufficient rate when the external pH is above 7.8, and it needs none or at most one when the external pH is below 7.8.

There is some genetic and physiological evidence regarding $K^+/H^+$ and $Na^+/H^+$ antiporters playing a major role in keeping the $pH_i$ constant. Two models for explaining the pH-homeostasis in E. coli, one involving $Na^+$ (18, 147) and the other involving $K^+$ (18, 23), have been proposed. However, the existing information is yet insufficient to provide evidence for or against either of these two models (18).

There are three major objections raised against the $Na^+/H^+$ antiport model for the pH regulation of cytoplasm. First, if the $Na^+/H^+$ antiport is so vital to life, there must be a sodium requirement for growth. However, in E. coli, sodium dependence for growth has not yet been reported. Nevertheless, it has been argued that all media are made in glassware and thus are contaminated (0.1 mM) with $Na^+$ that leaks from the glass (147). This argument was further substantiated by the finding of a $Na^+$-requirement in an alcalophile, B. firmus (106) and an alkalotolerant cyanobacterium, Synechococcus leopoliensis (126). Moreover, a very small amount (around 200 μM) of $Na^+$ was sufficient for the pH regulation of

cytoplasm in the $Na^+$ requiring B. firmus (106). The second criticism is that the model does not feature any entry route for $Na^+$ (except the symport with solutes). Considering the fact that an alkalophile is able to grow at alkaline pHs because of its highly active $Na^+/H^+$ antiport, which also makes it unable to grow at acidic pH (because of the excessive acidification of the cytoplasm) (105) and that an alkalotolerant cyanobacterium requires $Na^+$ for growth and pH regulation (126), there ought to be an active entry system for $Na^+$. Such a system should be electroneutral, otherwise $Na^+$ entry will collapse the $\Delta\psi$. As yet no such entry system has been identified. The third argument against the model arises from the fact that the $Na^+/H^+$ antiport mutants, besides being unable to grow at alkaline pH, are also deficient in at least two $Na^+$-linked transport systems — melibiose and glutamate. Thus the inactivation of yet other unknown $Na^+$-linked transport system(s) may be the cause of their inability to grow at alkaline pH.

The other, $K^+/H^+$ antiport model for pH-homeostasis has been criticized mainly because it requires an electroneutral uptake of $K^+$ and all the known $K^+$-transport systems known so far are electrogenic (103, 200). Another objection which has been raised against this model comes from the finding that the inability of the $K^+/H^+$ antiport mutants to grow at alkaline pH, can be overcome by the addition of some amino acids in the medium (mentioned in 18). Another remark against this model is that the $K^+$ concentration, known to be involved in controlling the osmotic pressure of the cell, seems unlikely to be

also involved in another important function of the cell — the $pH_i$ regulation (18).

Booth and Kroll (18) have recently suggested that the anion fluxes (e.g. $HCO_3^-$) may also be involved. The mechanism of pH-homeostasis of cytoplasm seems far from being well comprehended and more work is needed.

The $\Delta$*cya* mutant studied here is also sensitive to alkaline pH. The evidence presented in this thesis indicates that it can maintain a normal $\Delta$ pH but has a lowered $\Delta\psi$. This leads to the suggestion that cAMP has a role in the development and/or maintenance of the electrical gradient across the cytoplasmic membrane.

# MATERIALS AND METHODS

## Strains Used

All the bacterial strains used for this study are derivatives of *Escherichia coli* K-12. Their relevant characteristics are listed in Table 1.

## Media Used

Minimal medium: The minimal medium used contained 0.54% $K_2HPO_4$, 1.26% $KH_2PO_4$, 0.2% $(NH_4)_2SO_4$, 0.2% $MgSO_4 . 7H_2O$ and 0.001% $CaCl_2$ in distilled water. The pH of the medium was adjusted according to the requirement of the experiment by either HCl or KOH (10% stock solution) or NaOH (2.5N stock solution). Auxotrophic requirements were met by adding 30 ug/ml of the appropriate amino acid(s). Growth substrates were added to a concentration of 0.5%.

Luria broth: Luria broth contained 0.5% yeast extract, 0.5% NaCl, and 1% tryptone in distilled water.

Luria broth agar plates: Luria broth and 2% agar.

MacConkey agar plates: MacConkey agar plates contained standard MacConkey ingredients supplemented with 1% of the required carbon source(s) (lactose, maltose and arabinose). Whenever needed, 0.1 ml of a filter sterilized solution of cAMP (0.05M) was spread over the agar.

Tetrazolium agar plates: Tetrazolium agar plates (144) contained 25.5 gm of Antibiotic Medium #2 (from Difco) and 50 mg of 2, 3, 5-triphenyl tetrazolium chloride and 10 gm of the required carbon source (lactose or arabinose) in one litre of distilled water.

Table 1

List of bacterial strains and their relevant characteristics

| Strain | Genotype and/or Relevant Characteristics | Source |
|---|---|---|
| 1) CU1008 | *ilvA* deletion. | L.S. Williams |
| 2) NA-1 | *metB*; CU1008 derivative. | This laboratory |
| 3) JB-1 | $\Delta$*cya*, *ilvA*::Tn5, KanR. | J.Beckwith |
| 4) NAB-1 to 3 | $\Delta$*cya*, *ilvA*::Tn5, KanR, pHS; transductant of NA-1 using JB-1 as donor, selecting KanR. | This work |
| 5) K-10 | Wild type. | A. Garen |
| 6) NAB-1-T | *cya*$^+$, *ilvA*$^+$, KanS, pHR; transductant of NAB-1 using K-10 as donor, selecting *ilv*$^+$. | This work |
| 7) NABR-1 to 4 and NABR-A to D | *crp**, pHR; derivative of NAB-1 obtained by selecting for pHR. | This work |
| 8) 4506 | *cysG*. | CGS#4506 |
| 9) 4506-$\Delta$1 to 7 | $\Delta$*cya*, *ilvA*::Tn5, KanR, pHS; transductant of 4506 using JB-1 as donor, selecting for KanR. | This work |
| 10) 4506-$\Delta$1R to 4R and 4506-$\Delta$AR to DR | *cysG*$^+$, *crp**, *cya*$^+$, pHR; transductant of 4506-$\Delta$1 using NABR-1 as donor, selecting *cys*$^+$. | This work |
| 11) CA8001 | *thi*, *relA*. | J. Beckwith |
| 12) EC8439T | $\Delta$*lac*$^-$*pro*$^-$, *rpsL*, *supE*, *crp* $\Delta$39. | J. Beckwith |
| 13) CA8306 | $\Delta$*cya* derivative of CA8001. | J. Beckwith |
| 14) CA8303 | $\Delta$*cya* derivative of CA8001. | J. Beckwith |
| 15) 4506-W | cysteine independent transductant of 4506 using EC8439 as donor. | This work |
| 16) 4506-$\Delta$crp | $\Delta$*crp*, *cysG*$^+$ transductant of 4506 using EC8439 as donor. | This work |
| 17) CA8445 | *cya* $\Delta$8306, *crp*$^{\Delta 45}$, *rpsL*. | J. Beckwith |
| 18) CA8496 | $\Delta$*cya*, *crp* $\Delta$96. | J. Beckwith |

Note: CGS = Genetic Stock Centre

## Transductions

All transductions were done with phage P1CM (179) as described by Miller (127). Phages were prepared in superbroth (77). Transduction plates were incubated at 30°C for 2 days when rich medium was used and for 4 to 5 days whenever minimal medium was used. The transductants were purified and tested further for unselected characters.

A modified method of transduction was used when selection was made for the presence of transposon Tn5 KanR (15). These transposon-carrying transductants were selected for kanamycin resistance on Luria broth agar plates that were supplemented with 0.002% of kanamycin sulphate.

## Criteria For Adenyl Cyclase Mutants (cya)

The cya mutants were selected by their ability to utilize multiple carbon sources (maltose, lactose and arabinose) only when supplemented with exogenous cAMP. Since these mutants cannot use these sugars, they appear as white (non-fermenting) colonies on MacConkey indicator plates and as red (non-fermenting) colonies on tetrazolium indicator plates. The sugar non-fermenting colonies were able to ferment these sugars when the indicator plates were supplemented with cAMP and thus they appeared as dark pink colonies on MacConkey plates and as white colonies on tetrazolium plates. Strain NAB-1, used for further study, was also tested for growth on minimal medium, both liquid and solid, supplemented with lactose or maltose or arabinose in the presence and absence of exogenous cAMP.

Determination Of Growth Rate

Growth rate determinations were made by measuring the optical density at approximately 420 nm (purple filter) of the growing cultures using a Klett Summerson photoelectrocolorimeter. To ensure aerobic conditions all cultures were grown with vigorous shaking in a Gyrotary water bath shaker (from New Brunswick Scientific model #G76). Overnight cultures, grown with appropriate substrates, were harvested and resuspended to an O.D. of 20 Klett units in 20 ml of fresh sterile medium in 250 ml Erlenmeyer flasks fitted with side arms for direct reading of the optical density in a photoelectro-colorimeter. Optical density was measured at intervals of 30 minutes. The doubling time was determined from plots of optical density against time.

Determination Of Yield

The yield of protein per unit of carbon provided was determined by a previously described method (142). Each experiment was done in triplicate at two concentrations of the limiting carbon source, 0.04% and 0.08%. Overnight cultures, grown with the appropriate substrate, were used to inoculate 20 ml of fresh sterile minimal medium containing a limiting amount of carbon source. Parallel cultures were grown in side-arm-flasks to measure turbidity, in order to determine the time at which cultures entered a stationary phase, as judged by the constancy of optical density (due to the exhaustion of the carbon source). The cultures were then chilled and centrifuged at 4°C. The

harvested cells were treated with 5% TCA solution to precipitate protein which was then dissolved in NaOH and assayed.

## Assay For Protein

All the protein measurements were done by the method of Lowry et al. (112). Bovine serum albumin was used as a standard.

## Determination Of The Effect Of pH Shift On Growth

Cultures were grown aerobically at 37°C in 20 ml of minimal medium of desired pH with glucose as an energy source. Erlenmeyer flasks (250 ml capacity) fitted with sidearms were used. Optical density (O.D.) of the culture was measured every 30 minutes as described for the growth rate measurements. At an O.D. of approximately 50 Klett units, the pH of the medium was shifted quickly by adding an appropriate amount of HCl (10% stock solution) or NaOH (2.5 N stock solution) and measuring the pH of the culture. After the pH-shift, optical density was measured every 20 minutes. Controls were done by adding similar volumes of minimal medium or NaCl solution, to check whether the addition of fluid itself or $Na^+$ or $Cl^-$ could affect the growth rate.

## Assay Of ATPase Activity

Adenosine triphosphatase activity was measured in membrane preparations using a combination of the previously described methods (21, 49, 73, 143). Cultures grown aerobically on glucose minimal

medium at 37°C, were chilled and harvested in mid-exponential phase using the method described by Evans (49). Cells were washed with 0.15 M Tris-HCl (pH 6.4) containing 1.0 mM of ethylenediamine tetraacetic acid (EDTA) and 0.6 M sucrose. Cells were then resuspended in the same buffer to a final volume that is one fourth of that of the original culture. Lysozyme (20 μgm/ml) was added to this cell suspension and the suspension was incubated at 37°C for 75 minutes. Spheroplasts were harvested by centrifugation at 10,000 rpm for 20 minutes in an IEC B20 centrifuge with an 8 x 50 rotor, washed with the same cold buffer-sucrose solution (pH 6.4) and centrifuged again for 10 minutes at 10,000 rpm. The pellet was resuspended in approximately two thirds of the original (culture) volume of cold 0.02 M Tris-HCl (pH 7.5), and centrifuged at 20,000 rpm in a Beckman Ultracentrifuge (Model #L8-70) for 20 minutes. The pellet obtained from this centrifugation was washed three times with 0.02 M Tris-HCl (pH 7.5) containing 1.0 mM $MgCl_2$, and resuspended at 10-15 mg protein per ml. The ATPase activity was assayed by measuring the production of inorganic phosphate ($P_i$) per minute per mg of protein using ATP as a substrate (143).

Measurements Of The Rate Of Oxygen Uptake By Cells And Membrane Vesicles

Oxygen uptake was determined by the method of Barnes and Kaback (13) using a Clark oxygen electrode (138) fitted with a recorder. The apparatus was calibrated with air saturated water at 37°C. The value

of $O_2$ in the air-saturated water was taken as 225 n moles per ml (66).

Cells were harvested from exponentially growing cultures, washed and resuspended in minimal medium at pH 7.0. The total volume of the reaction mixture used was 2 ml, which consisted of 1.8 ml of buffer at appropriate pH, 0.1 ml of cell or vesicle suspension and 0.1 ml of the energy source. A 20% stock solution of glucose was used for the whole cell assays. The same method was followed for the assays done on vesicles except that instead of glucose, 20 µl of 0.1 M lactate was used as energy source.

Preparation Of Membrane Vesicles

Membrane vesicles were prepared by using a previously described method (87). Nine liters of culture was grown in a Labrofermentor from New Brunswick Scientific Co. (Model #FS-305) at 37°C, with vigorous stirring to ensure aerobic conditions. The cells were harvested at room temperature by passing the culture through multitube porcelain filters (from New Selas Corporation) and then, by centrifuging the concentrated bacterial suspension to obtain a compact pellet. The cells were then washed with 10 mM Tris buffer (pH 8) and resuspended in 30 mM Tris HCl (buffered at pH 8) containing 20% sucrose, using 50 ml of buffer per gm of cell. The cell suspension was then incubated with 10mM of ethylenediamine tetraacetic acid (EDTA) for about 15 minutes at 37°C. After this incubation period, cells were treated with lysozyme (50 mg per liter) for 45 minutes to loosen the cell wall. The spheroplasts were then centrifuged and

resuspended in 20 ml of 0.1 M phosphate buffer (pH 6.6) containing 20% sucrose and $MgSO_4$ (20 mM). Twenty-five mg of RNase and 25 mg of DNase were then added. The cell suspension was immediately added to 1.5 liters of potassium phosphate buffer (50 mM, pH 6.6) with constant stirring of the suspension and incubated for 15 minutes at 37°C. After this incubation, EDTA was added at a concentration of 10 mM. After incubation for another 15 minutes, $MgSO_4$ was added to a concentration of 15 mM. After 15 more minutes of incubation, the suspension was chilled, centrifuged for 10 minutes at 12,000 rpm and resuspended in approximately 20-40 ml of 50 mM potassium phosphate buffer at pH 6.6. The preparation was inspected for the number of whole cells as compared to vesicles by phase contrast microscopy. One to three whole cells were observed under the microscopic field using oil immersion, and the preparation was considered to be satisfactory. The preparation was then distributed into Eppendorf tubes (1.5 ml capacity), frozen quickly (using liquid nitrogen) and stored at -65°C. When needed, the vesicles were thawed quickly under warm running water.

Assay Of Active Amino Acid Transport

The assay was performed in glass tubes containing small magnetic stirring bars and fitted with aeration devices that delivered water saturated air. The tubes were kept in a water bath set at 25°C on a magnetic stirring plate.

For assays done on cells, the cells were grown in glucose minimal

medium (pH 6.4), chilled and harvested at mid-exponential phase by centrifuging for 10 minutes at 6,000 rpm. The cell pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.0), and washed three times with the same buffer.

The assay mixture contained 50 µl of 0.1 M potassium phosphate buffer at the desired pH, 20-30 µl of distilled water (to adjust the assay size to 100 µl), 10 µl of 0.1 M solution of $MgSO_4.7H_2O$ and 5 µl of cell suspension (approximately 0.02 to 0.05 mg protein) or membrane vesicles (approximately 0.1 to 0.2 mg of protein). Glucose was added to a final concentration of 0.5% and served as the energy source when assays were done on cells. For assays done on vesicles, sodium ascorbate and phenazine methosulfate (ASC-PMS) were used as energy source (100). For each assay, 10 µl of sodium ascorbate (17.6 mg per ml, neutralized with NaOH to pH 6.6-7.0) and 2 µl of a 10 mM solution of phenazine methosulfate were added to the reaction tubes. Approximately 30 seconds after the addition of the energy source, the transport reaction was started by adding 2 µl of $C^{14}$ labelled amino acid (final concentration 4 µM, radioactivity 0.11 µCi per ml). The reaction was terminated at 0, 1, 2, 3, and 5 minute intervals by diluting with 2 ml of 0.1 M lithium chloride solution and filtering quickly through a nitrocellulose filter (from Schleicher and Schuell, pore size 0.45 µM) that was attached with a vacuum device to separate cells from the medium. The filters were dried and radioactivity was counted in the scintillation fluid (0.5% PPO and 0.05% POPOP in toluene) using a LKB Wallac liquid scintillation counter.

Whenever needed, valinomycin at a final concentration of 5 μM (1 μl of 0.05 mM stock solution)and nigericin at a final concentration of 0.5 μM (1 μl of 50 μM stock solution) was added to the assay mixture just before the addition of labelled proline. From this point on, the procedure for this experiment was as described previously. Since these antibiotics were dissolved in absolute ethanol, a control was done by adding 1 μl of ethanol to the assay in the absence of antibiotics and no effect was observed.

The rate of transport of labelled amino acid was determined by calculating cpm uptake per minute by each sample and averaging all the determinations. The linear part of the curve was taken as a measure of initial rate of uptake of amino acid. The rate of uptake has been expressed as n moles of amino acid taken in per minute per mg of protein by converting the cpm of labelled amino acid into n moles.

Measurement Of Proton Gradient ($\Delta$pH) In The Membrane Vesicles

The proton gradient across the membrane was determined from the distribution of $^{14}C$ labelled sodium benzoate, following the method of Kroll and Booth (103) with some modifications. Vesicles were used instead of cells and the energy source used was ascorbate - phenazine methosulfate (ASC-PMS). The assay mixture was incubated under aerobic conditions at 25°C in 50 ml flasks shaken vigorously in a water bath. The assay mixture contained 500 μl of potassium phosphate buffer (400 mM) at the desired pH, 200 μl of distilled water, 100 μl of $MgSO_4.7H_2O$ (0.1M), 200 μl of vesicles, 100 μl of sodium ascorbate

solution (17.6 mg per ml, pH 6.6-7.0), 10 µl of PMS (10 mM) and 2 µl of catalase (25 mg per ml). Four and twelve µl (stock solution 16 µCi/ml, 600 µM) of the radioactive probe, $^{14}C$ sodium benzoate, were added to get a final concentration of approximately 2 and 6 µM (0.05-0.16 µCi per ml) respectively. After 5 min of incubation, 500 µl of the assay mixture were taken out into an Eppendorf tube (1.5 ml capacity) that contained 10 µl of 6% hydrogen peroxide and centrifuged quickly at 12,000 g for 20 seconds in an Eppendorf centrifuge from Brinkmann Instruments (Model #5414). During the centrifugation process, aerobic conditions were maintained due to the production of oxygen from the reaction between catalase and $H_2O_2$ present in the sample. One hundred µl of the extra-vesicular fluid (supernatant) were transferred into another Eppendorf tube that contained 200 µl of 0.1 M potassium phosphate buffer and a non-radioactive vesicle pellet (obtained from a parallel set of experiments in which the labelled probe was not added). This suspension was used in measuring the radioactivity in the extracellular fluid. The remaining supernatant was aspirated from the pellet and the pellet was resuspended in 300 µl of buffer. Two hundred µl of these suspensions were added to 5 ml of Bray's scintillation fluid (22) and the radioactivity was counted.

The volume of extracellular fluid trapped in the pellet was determined in a parallel set of experiments in which sodium benzoate was replaced by 12 µl of $^{3}H$ labelled polyethylene glycol (PEG) (final concentration - 0.34 mM, radioactivity - 0.83 µCi per ml). PEG does

not penetrate the membrane of the vesicle and thus is used to determine the trapped extracellular fluid in the pellet (193).

Measurement Of Electrical Gradient ($\Delta\psi$) In The Membrane Vesicles

The electrical gradient was measured using the distribution of $TPP^+$ (tetra phenyl phosphonium ion), a lipophilic cation, that distributes itself according to the electrical charges on the two sides of the membrane. The assay protocol was similar to the one described for the measurement of $\Delta$pH, except that the buffer used was at a concentration of 100 mM (instead of 400 mM) and the labelled probe was $^3$H-TPPBr. Six, twelve and eighteen $\mu$l of $^3$H-$TPP^+$ (100 $\mu$M, 17 $\mu$Ci/ml) were added to get a final concentration of 0.5 to 1.5 $\mu$M. Non-specific binding of $TPP^+$ was calculated from the vesicle associated radioactivity in the absence of the energy source. Sampling and subsequent procedures were as described for the measurement of $\Delta$pH.

Measurement Of Proton Gradient ($\Delta$pH) In The Non-growing, Respiring Cells

Cells from overnight cultures were inoculated into fresh medium at the same pH as that used in the experiment, harvested in mid-exponential phase (optical density at $A_{625nm}$ = 1.0) by centrifugation at 4°C for 10 minutes at 6,000 rpm, and suspended in either 50 mM MesTham pH 6.0 (when grown at pH 6.0) or 50 mM Tris Tham pH 8.3 (when grown at pH 7.8), to a cell density of approximately 10

at $A_{625nm}$.

The proton gradient across the membrane was determined by using a combination of methods used by Kashket (94), and Kroll and Booth (103). The assay mixture (total volume 6 ml) containing cells (at $A_{625nm}$ = 1.0), a buffer of the desired pH, 0.8 ml of 10% glucose, 1 mM sodium phosphate (pH 7), EDTA (10 mM for assays done at pH 8.3 and 30 mM for assays done at pH 6.0) and the radioactive probe, was incubated for 15 minutes with rapid shaking at 28°C. During the last minute of the incubation period, 60 µl of catalase (25 mg/ml) was added to the assay mixture. After the 15 minutes of incubation, samples of 0.8 ml were transferred to Eppendorf tubes (size 1.9 ml) containing 16 µl of $H_2O_2$ (20%), and centrifuged for 2 minutes in an Eppendorf centrifuge. Fifty µl of the supernatant from each tube and the pellet were counted for radioactivity, using Bray's scintillation fluid. Samples (size 1 ml) were also drawn to measure the external pH. The volume of the extracellular fluid trapped in the pellet was measured in parallel cultures in which sodium benzoate was replaced by $^3$H-PEG.

Measurement Of Electrical Gradient ($\Delta\psi$) In Non-growing, Respiring Cells

For measuring the electrical gradient, the assay protocol was similar to the one used for the measurement of $\Delta$pH in the cells, except that the final volume of the assay mixture was 8 ml and the labelled probe used was $^3$H-TPP$^+$. One hundred µl of $^3$H-TPP$^+$ (0.07 µCi/µl) was added to get a final concentration of 1.25 µM.

Various concentrations of EDTA were used at each assay pH, to determine the concentration necessary to give maximal $^{3}H$-$TPP^{+}$ uptake. For cultures growing at pH 6.0 and pH 8.2 respectively, 20-30 and 5-15 mM of EDTA gave maximum uptake of the probe. Hence, in all other assays 30 and 10 mM of EDTA were used for measurements made at pH 6 and 8.2, respectively. For the measurement of the non-specific binding of the probe, samples of 2 ml were taken out from the assay mixture, mixed with 0.1 ml of butanol and incubated for 30 minutes at 36°C. After the incubation, cells were vortexed, kept at room temperature for 5 minutes and samples of 0.8 ml were taken out in Eppendorf tubes. The samples were then centrifuged for 2 minutes and 50 µl of the supernatant and the pellet were counted for radioactivity using Bray's scintillation fluid.

Calculation Of $\Delta\psi$ And $\Delta$pH

Calculations of $\Delta\psi$ and $\Delta$ pH values were made using the Nernst equation (17, 92, 119). The intracellular concentration of the probes in the vesicles was calculated using a value of 2.2 µl of intracellular fluid per mg of membrane protein (173). The intracellular concentration of probes in the cells was calculated by using a value of 0.624 µl of intracellular fluid per ml of cell suspension at an optical density of one at $A_{625nm}$.

For the calculation of $\Delta$ pH the equation used was:

$$\Delta pH(mV) = 59 \log\left(\frac{\text{cpm in each benzoate pellet - extracellular cpm bound to the pellet}}{\text{Intracellular cpm at equilibrium}}\right)$$

where,

the intracellular cpm at equilibrium = cpm/$\mu$l of benzoate supernatant x intracellular volume ($\mu$l)

and,

the extracellular cpm bound to the pellet = cpm/$\mu$l of benzoate supernatant x volume ($\mu$l) of contaminating extracellular fluid,

where,

the volume ($\mu$l) of the contaminating extracellular fluid = $\frac{\text{cpm in each PEG pellet}}{\text{cpm/}\mu\text{l of PEG supernatant}}$

For the calculation of $\Delta\psi$ the equation used was:

$$\Delta\psi(mV) = 59 \log\left(\frac{\text{cpm in each } TPP^+ \text{ pellet - cpm bound non-specifically to the pellet}}{\text{Intracellular cpm at equilibrium}}\right)$$

where,

the intracellular cpm at equilibrium = cpm/$\mu$l of $TPP^+$ supernatant x intracellular volume ($\mu$l),

the cpm bound non-specifically to the pellet from the vesicles = cpm in each pellet from assays done without energy source

and,

the cpm bound non-specifically to the pellet from the cells = cpm in each pellet from the butanol treated cells.

# RESULTS

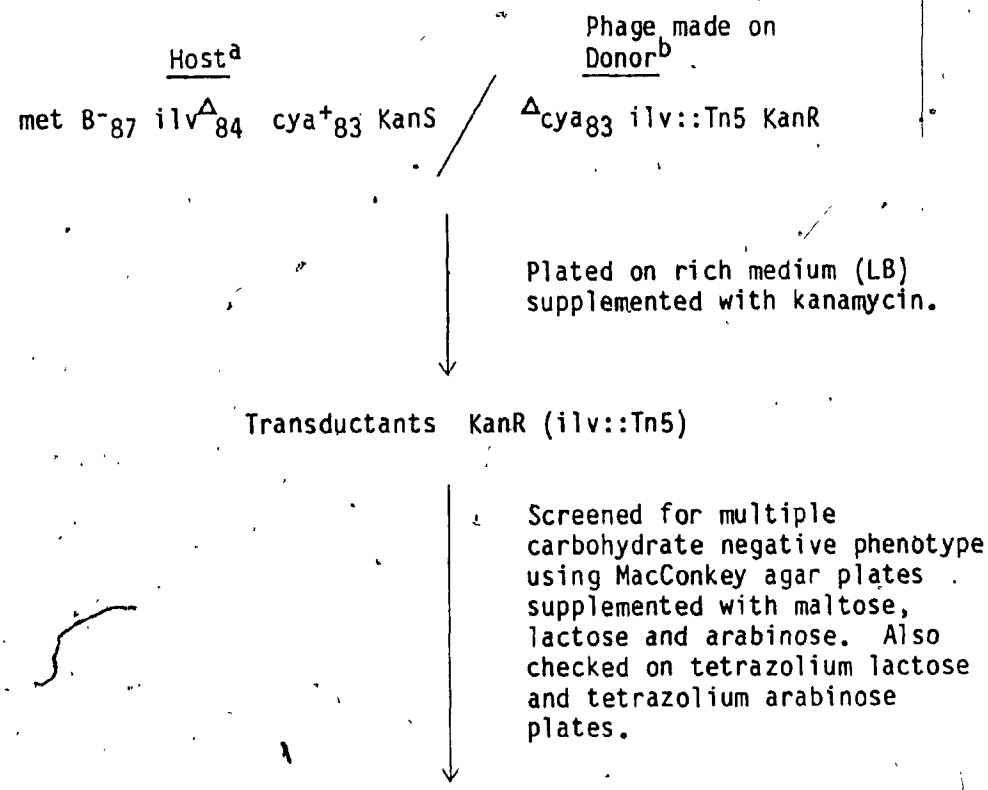
## I. Transduction Of A Known Adenyl Cyclase Deletion ($\Delta$cya) Into Strain NA-1:

Since the purpose of this study was to investigate the general effects of an adenyl cyclase mutation (cya) on metabolism, a strain carrying a well characterized adenyl cyclase deletion mutation was obtained from Dr. J. Beckwith and the mutation was transferred into our regular laboratory strain. The method used to do this depended on having a donor strain carrying the transposon Tn5, specifying kanamycin resistance, inserted into a gene near the cya locus and screening kanamycin resistant transductants for those that also carry the cya mutation.

The adenyl cyclase deletion mutant (strain JB-1), used as a donor, carried a Tn5 inserted into the ilvA gene, which is approximately one minute away from the cya locus (11). Strain JB-1 was therefore an isoleucine-requiring, kanamycin-resistant $\Delta$cya strain. The recipient (strain NA-1), a derivative of the strain CU1008 (Table 1), required methionine and isoleucine as it carried metB and ilvA mutations. Phage P1CM was grown on the donor strain (JB-1) and a selection was made for kanamycin-resistant transductants by plating on rich medium (Luria Broth) supplemented with kanamycin.

The scheme of the experiment is given in Figure 2. The kanamycin resistant transductants were screened for the cya mutation by testing

Figure 2. Scheme of transduction of $^{\Delta}$cya mutation from JB-1 ($^{\Delta}$cya) strain to NA-1 ($cya^{+}$) strain.




[a]Host strain used is NA-1

[b]Donor strain used is JB-1

Note: The numbers after the gene symbols represent map locations. The symbol :: means Tn5 is inserted into the gene preceding the symbol.

on MacConkey plates supplemented with lactose, maltose and arabinose, with and without cyclic adenosine monophosphate (cAMP). Transduetants that were white (pleiotropic carbohydrate negative) on the MacConkey plates and dark pink (pleiotropic carbohydrate positive) on the MacConkey plates supplemented with exogenous cAMP, were considered to be $^{\Delta}$cya strains.

Seven hundred kanamycin resistant transductants were selected in each of two separate experiments. For the first experiment, 38 of the kanamycin resistant transductants were purified and one proved to be $^{\Delta}$cya (Table 2).

In the second experiment, two of the seventy-three transductants purified carried $^{\Delta}$cya . These three strains (NAB-1, -2, and -3) were all able to grow at the expense of maltose, lactose and arabinose, if cAMP was also supplied.

These pleiotropic carbohydrate negative strains were further characterized as $^{\Delta}$cya by their behaviour on tetrazolium indicator plates with lactose and arabinose, on which they produced colonies which were red (pleiotropic carbohydrate negative phenotype) in the absence, and colourless (pleiotropic carbohydrate positive phenotype) in the presence, of cAMP. Growth in minimal medium with maltose, lactose or arabinose depended on the addition of cAMP.

The frequency of cotransduction of ilvA and cya loci was lower than expected (60%, 35) for genes so close to each other. Two factors might be the cause for this. Firstly, that the ilvA gene carried an

Table 2

Transduction frequency of loci ilv::Tn5 and cya

| Transduction # | Relevant Genotype | | Selected Marker[a] | Unselected Marker[b] | Percentage of Strains Carrying Unselected Donor Marker |
|---|---|---|---|---|---|
| | Host | Donor | | | |
| (1) | NA-1 ilv⁻ cya⁺ KanS | JB-1 ilv::Tn5 KanR Δcya | ilv::Tn5 (KanR) | Δcya | 1/38 (2.6%) |
| (2) | ilv⁻ cya⁺ KanS | ilv::Tn5 KanR Δcya | ilv::Tn5 (KanR) | Δcya | 3/73 (4.2%) |

Note: [a]: KanR scored as ability to grow on medium supplemented with kanamycin.

[b]: Δcya scored on MacConkey plates (see text).

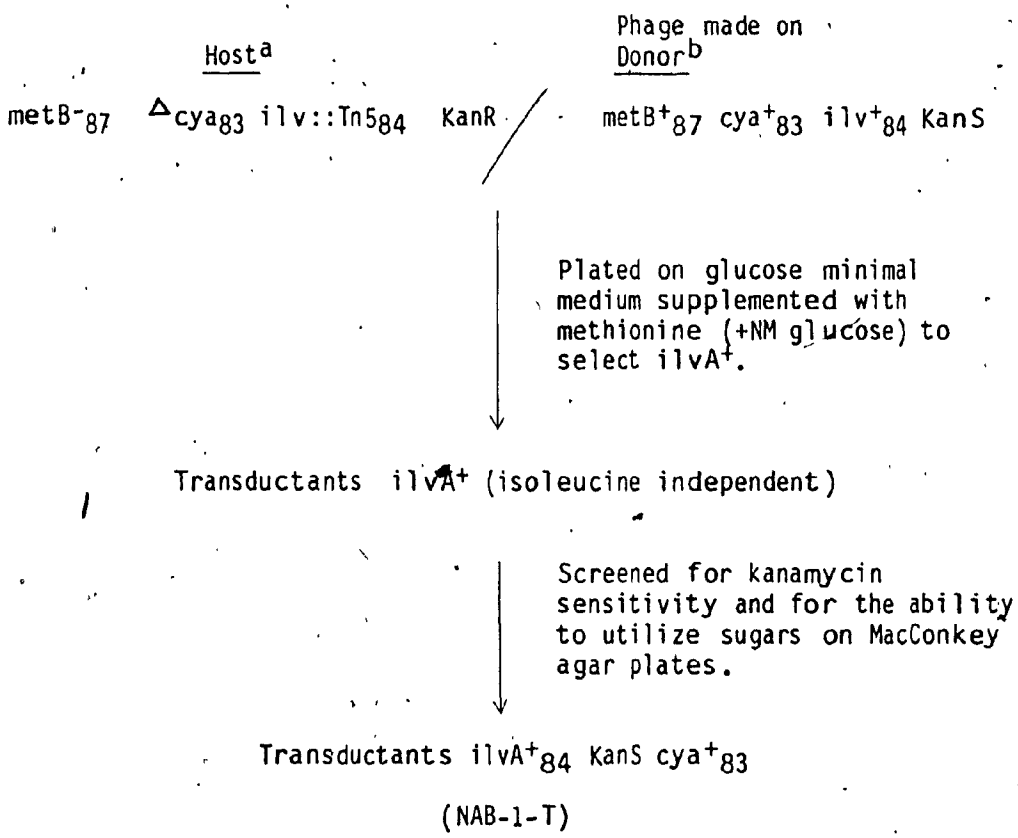
insertion (Tn5) which increased the distance between the *ilvA* and *cya* loci on the chromosome. Secondly, that the *cya* mutants grow very poorly and therefore might be hard to find among the $cya^+$ transductants on the plate. A third reason could be use of an *ilvA* deletion mutant as recipient which might have favoured selection of recombinants where genes on the opposite side of *ilvA* from *cya* were transduced.

## II. Genetic Evidence That The Adenyl Cyclase Gene Has Been Deleted From Strain NAB-1:

The pleiotropic carbohydrate non-utilizing strain (NAB-1) described in the preceding section was selected for further study. It was constructed through transduction and was tested for the phenotypic characteristics of a *cya* mutant strain. Since this strain was to be used in many of the following experiments, it was important to be certain that it did in fact carry a $^{\Delta}$*cya* mutation. If this strain in fact carries a *cya* deletion and a transposable element at the *ilvA* locus, it should be possible to co-transduce the *ilvA* and *cya* loci into this strain from a wild type donor strain. To confirm that a selection for ilvA$^+$ transductants will also co-select cya$^+$, strain NAB-1 was transduced with P1CM phage grown on strain K-10, selecting for isoleucine independent transductants. The scheme of this transduction is given in Figure 3. About 700 ilvA$^+$ transductants were selected. All were kanamycin-sensitive as would be expected, since the strain NAB-1, during the transduction of ilvA$^+$ locus from the donor (K-10), has to lose the transposable element (Tn5$^{kan}$R) that was inserted in the *ilvA* gene. Of 58 ilvA$^+$ transductants studied further, 30 were cya$^+$, ie., were able to utilize lactose, maltose and arabinose (Table 3).

These results prove that the multiple carbohydrate non-utilizing strain constructed in the preceding section does carry a mutation at the *cya* locus. This is linked to the *ilvA* locus with a linkage value of 51.7% (Table 3), (11, 35).

Figure 3. Scheme of transduction for testing the presence of cya deletion in strain NAB-1 by linkage to ilvA.




[a]Host strain used is NAB-1

[b]Donor strain used is K-10

Note: The numbers after the gene symbols represent map locations.

Table 3

Transduction frequency of loci ilvA and cya in the transduction between strains NAB-1 and K-10

| Relevant Genotype | | Selected Marker[a] | Unselected Marker[b] | Percentage of Strains Carrying Unselected Donor Marker |
|---|---|---|---|---|
| Host | Donor | | | |
| NAB-1 ilvA::Tn5 KanR Δcya | K-10 ilv+ KanS cya+ | ilv+ (KanS) | cya+ | 30/58 (51.7%) |

Note: [a]: ilvA+ scored as ability to grow on minimal medium without isoleucine and valine.

[b]: cya+ scored on MacConkey plates (see text).

## III. Utilization Of Various Carbon Sources By The Mutant (NAB-1) And The Parent (NA-1) Strains:

Adenyl cyclase mutants are unable to grow on many substrates commonly used by *E. coli*, and grow slowly on most of the ones they do use. This slow growth of *cya* mutants has been treated previously as a problem in sugar transport, the suggestion being made that a $\Delta$*cya* mutant will grow slowly on sugars transported by the phosphoenol-pyruvate transport system (PTS) and not at all on sugars transported in other ways (50, 163). Studies with strain NAB-1 did not support this generalization. Strain NAB-1 grew slowly on two PTS-sugars tested, glucose and glucosamine (Table 4). However, it grew on the non-PTS substrate, glycerol, almost as rapidly as the parent strain. It also grew, though slowly, on L-alanine, D-alanine, gluconate and pyruvate, but not on ribose and melibiose.

The $\Delta$*cya* mutant was unable to use any sugar which is degraded by catabolite-repressed systems (lactose, arabinose, maltose). It grew slowly on those sugars which it can use (except glycerol), but this does not seem to be due to a defect in a particular type of transport system.

Table 4

Growth of strains NA-1 and NAB-1 on various PTS and non-PTS carbon sources

| Substrates | Strains | |
|---|---|---|
| | NA-1 (cya$^+$) | NAB-1 ($\Delta$cya) |
| (1) PTS substrates: | | |
| Glucose | ++ | + |
| Glucosamine | ++ | + |
| (2) non-PTS substrates: | | |
| D-alanine | ++ | + |
| L-alanine | ++ | + |
| Pyruvate | ++ | + |
| Gluconate | ++ | + |
| Glycerol | + | + |
| Melibiose | + | - |
| Ribose | + | - |

Note: ++ denotes extensive growth of overnight cultures.
+ denotes moderate growth of overnight cultures.
- denotes no growth of overnight cultures.

## IV. Effect Of Exogenous Cyclic Adenosine Monophosphate (cAMP) On The Growth Rate In Glucose Minimal Medium:

The mutant grew more slowly on glucose than the parent. In order to show that this decrease in growth rate is indeed due to a deficiency of cAMP, the effect of various concentrations of exogenous cAMP on the rate of growth was studied.

The doubling time, on glucose, for the mutant was 140 minutes, as compared to 60 for the parent strain (Fig. 4, 5; Table 5). Addition of cAMP drastically reduced the doubling time of the mutant, but did not significantly affect the doubling time of the parent, though it may have slightly increased the growth rate (Table 5). Exogenous cAMP at a concentration of $1.0 \times 10^{-4}$ M reduced the doubling time of the mutant to 90 minutes. At $2.5 \times 10^{-4}$ M the doubling time (65 minutes) of the mutant was close to that of the parent strain (60 minutes). The highest concentration of cAMP was inhibitory to both the parent and the mutant.

These results show that the slower growth rate of the mutant strain is due to the deficiency of cAMP as the addition of cAMP at a concentration of $1.0\text{-}2.5 \times 10^{-3}$ M returns the growth rate of the mutant to a near normal value.

Figure 4: The effect of various concentrations of exogenous cAMP on the growth of strain NAB-1 ($\Delta$cya) in glucose minimal medium.

Symbols: (a) cAMP concentration 0

(b) cAMP concentration $1.0 \times 10^{-4}$M

(c) cAMP concentration $2.5 \times 10^{-4}$M

(d) cAMP concentration $5.0 \times 10^{-4}$M

(e) cAMP concentraton $1.0 \times 10^{-3}$M

(f) cAMP concentration $2.5 \times 10^{-3}$M

(g) cAMP concentration $5.0 \times 10^{-3}$M

Note: The glucose concentration was 0.5%.
The pH of the medium was 6.4.
The temperature was 37°C.

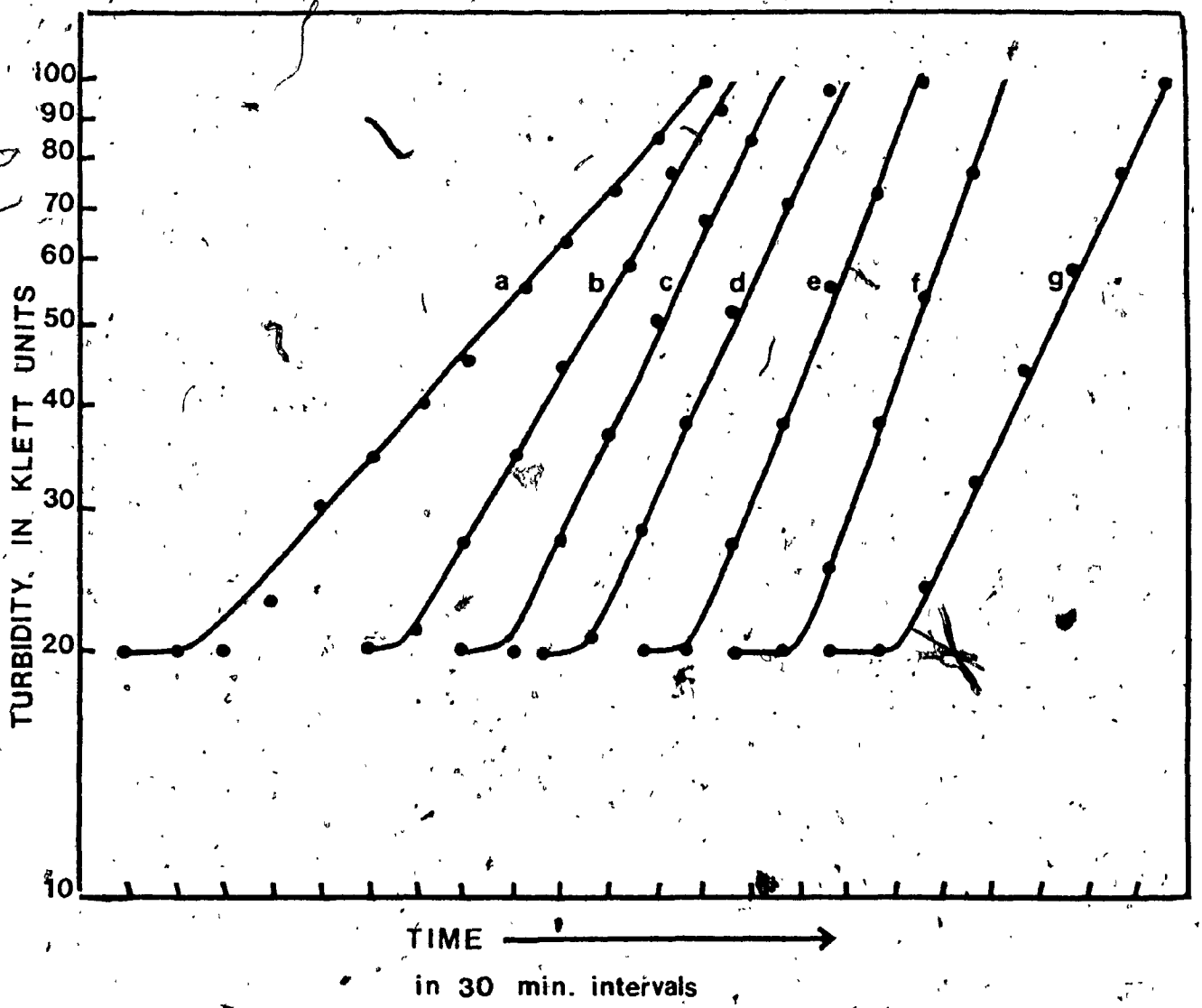

100
90
80
70
60
50
40
30
20
10
TURBIDITY, IN KLETT UNITS
a
b
c
d
e
f
g
TIME
in 30 min. intervals

FIGURE 4

Figure 5: The effect of various concentrations of exogenous cAMP on the growth of strain NA-1 ($cya^+$) in glucose minimal medium.

Symbols: (a) cAMP concentration 0

(b) cAMP concentration $1.0 \times 10^{-4}$M

(c) cAMP concentration $2.5 \times 10^{-4}$M

(d) cAMP concentration $5.0 \times 10^{-4}$M

(e) cAMP concentraton $1.0 \times 10^{-3}$M

(f) cAMP concentration $2.5 \times 10^{-3}$M

(g) cAMP concentration $5.0 \times 10^{-3}$M

Note: The glucose concentration was 0.5%.
The pH of the medium was 6.4.

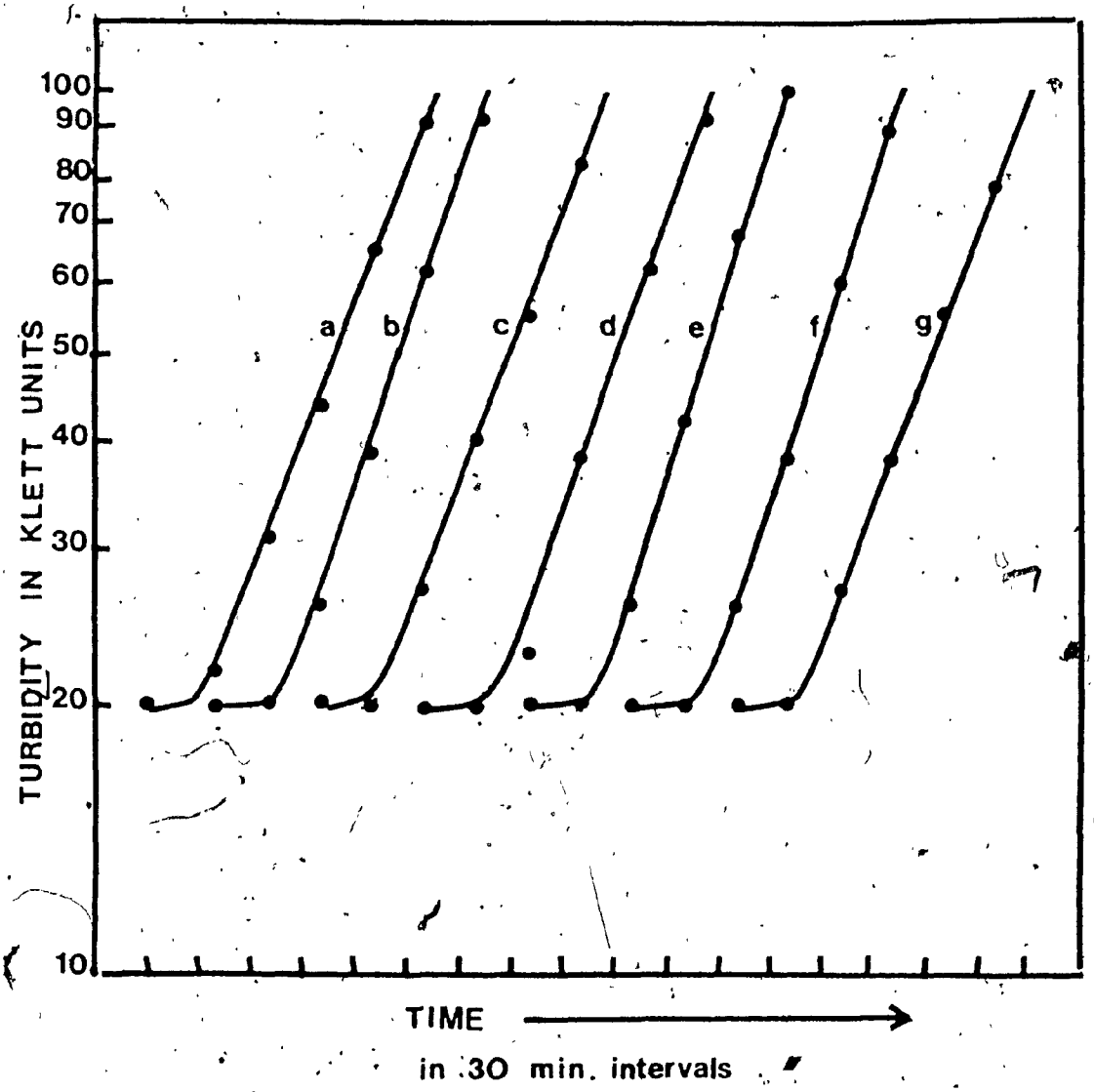

100
90
80
70
60
50
40
30
20
10
TURBIDITY IN KLETT UNITS
a
b
c
d
e
f
g
TIME
in 30 min. intervals

FIGURE 5

Table 5

The effect of various concentrations of exogenous cAMP on the doubling time on glucose of parent (NA-1) and mutant (NAB-1) strain

| Concentration of cAMP in the medium (M) | Doubling Time (in minutes) Strain NA-1 ($cya^+$) | Strain NAB-1 ($\Delta cya$) |
|---|---|---|
| (1) 0 | 60 | 140 |
| (2) $1.0 \times 10^{-4}$ | 55 | 90 |
| (3) $2.5 \times 10^{-4}$ | 55 | 65 |
| (4) $5.0 \times 10^{-4}$ | 50 | 65 |
| (5) $1.0 \times 10^{-3}$ | 60 | 60 |
| (6) $2.5 \times 10^{-3}$ | 60 | 55 |
| (7) $5.0 \times 10^{-3}$ | 70 | 80 |

Note: The glucose concentration was 0.5%.
The experimental temperature was 37°C and the pH of the medium was 6.4.

## V. Effect Of pH On The Growth Rate With Various Carbon Sources (Glucose, Glycerol and Pyruvate):

As shown in the previous section, the $\Delta$*cya* strain grew more slowly than the parent. This experiment was done at pH 6.4. The slow growth was even more evident at higher pH values.

When the mutant (strain NAB-1) was tested in a series of media buffered at pHs between 6.0 to 7.8, it grew faster at lower pH values, the doubling time increasing with the pH. That is, the mutant grew with a doubling time of 135 minutes at pH 6.0 and of 280 minutes at pH 7.8. The parent strain grew at about the same rate at pH values from 6.0 to 7.8, although it grew somewhat more slowly at pH 7.0 and above (apparent doubling time: 60 minutes at pH 6.0, 75 minutes at pH 7.8). The doubling time of the mutant strain at pH 6.0 was 135 minutes, while for the parent it was 60 minutes (Fig. 6, 7, Table 6). The doubling time increased gradually from 150 to 280 minutes as the pH value increased from 6.4 to 7.8.

On glycerol minimal medium the mutant grew slightly more slowly than the parent strain, the doubling time for the mutant being 90 minutes as compared to 80 minutes for the parent strain (Fig. 8, Table 7). The growth rate on glycerol was not significantly affected by the pH of the medium.

On pyruvate minimal medium the mutant grew more slowly than the parent strain and was also sensitive to the pH of the medium but the growth rate was faster than that on the glucose minimal medium

Figure 6: Growth of strain NAB-1 ($\Delta$cya) on glucose minimal medium as a function of pH.

Symbols: (a) pH 6.0

(b) pH 6.4

(c) pH 7.0

(d) pH 7.4

(e) pH 7.8

Note: The glucose concentration was 0.5%.
The temperature was 37°C.
The pH of the stock medium was 6.4.
The pH was adjusted by adding either HCl (10% stock solution) or NaOH (2.5 N stock solution).

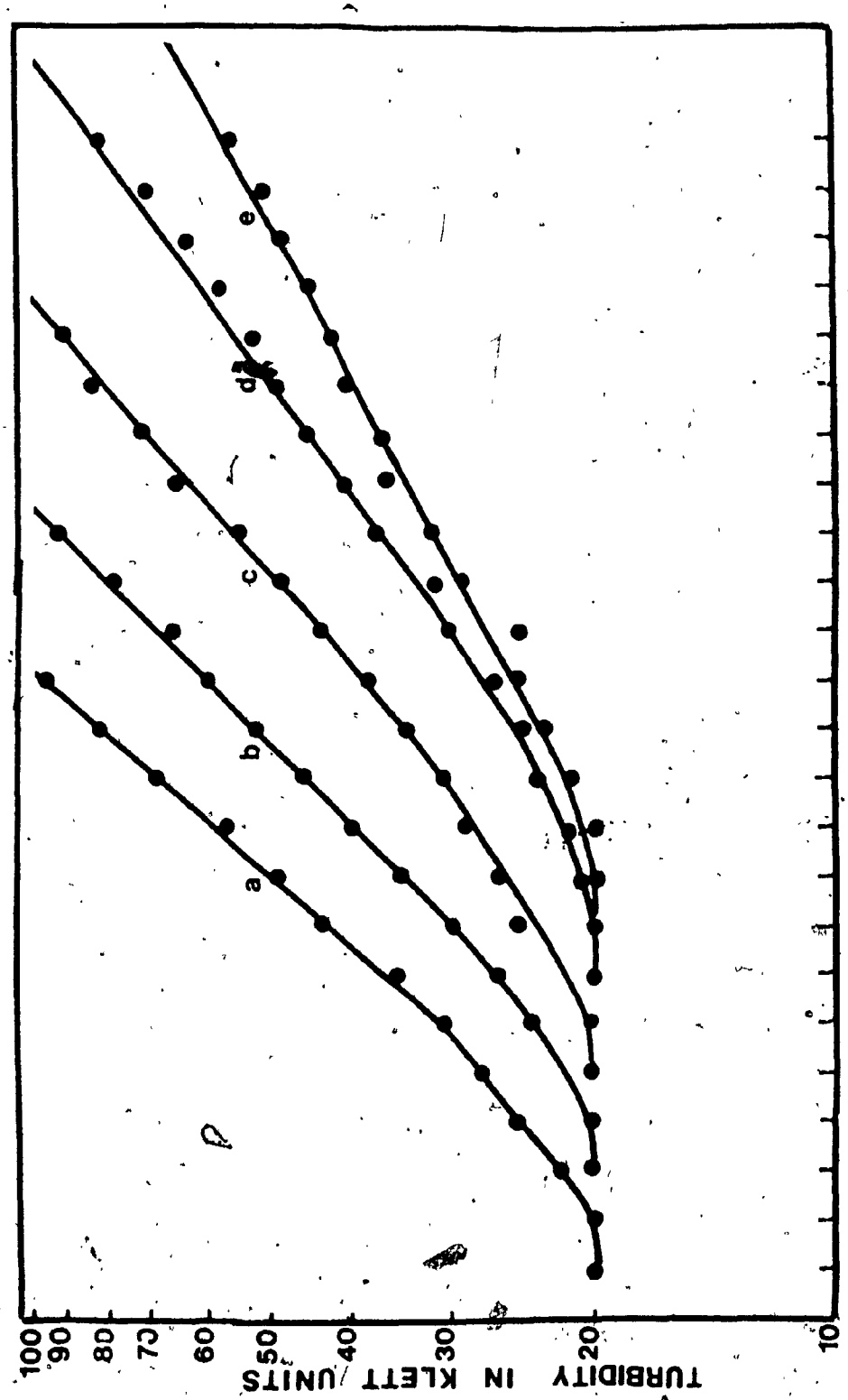

TURBIDITY IN KLETT UNITS
100
90
80
70
60
50
40
30
20
10
a
b
c
d
e
TIME in 30 min. intervals

FIGURE 6

Figure 7: Growth of strain NA-1 ($cya^+$) on glucose minimal medium as a function of pH.

Symbols: (a) pH 6.0

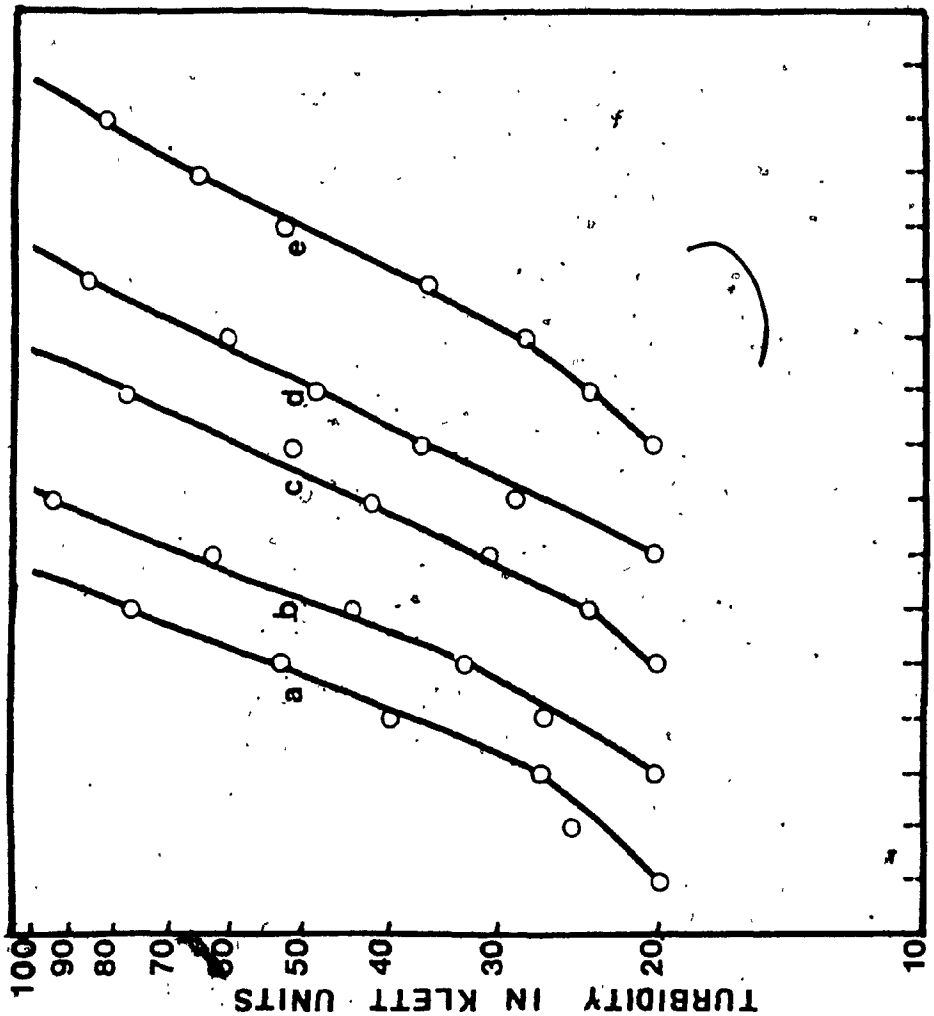
(b) pH 6.4

(c) pH 7.0

(d) pH 7.4

(e) pH 7.8

Note: The glucose concentration was 0.5%.
The pH of the stock medium was 6.4.
The temperature was 37°C.
The pH was adjusted by adding either HCl (10% stock solution) or NaOH (2.5 N stock solution).


TURBIDITY IN KLETT UNITS
100
90
80
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60
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30
20
10
a
b
c
d
e
TIME
in 30 min. intervals

FIGURE 7

Table 6

The effect of pH of the growth medium on the doubling time of mutant (NAB-1) and parent strain (NA-1) on glucose minimal medium

| | pH of the medium | Doubling Time (in minutes) in medium adjusted with HCl or NaOH* | | Mono- and dibasic Salts** | | KOH*** | |
|---|---|---|---|---|---|---|---|
| | | Strain NA-1 ($cya^+$) | Strain NAB-1 ($\Delta cya$) | Strain NA-1 ($cya^+$) | Strain NAB-1 ($\Delta cya$) | Strain NA-1 ($cya^+$) | Strain NAB-1 ($\Delta cya$) |
| (1) | 6.0 | 60 | 135 | 58 | 130 | 63 | 135 |
| (2) | 6.4 | 58 | 150 | 55 | 150 | 58 | 160 |
| (3) | 7.0 | 69 | 180 | 60 | 190 | 60 | 170 |
| (4) | 7.4 | 72 | 210 | 60 | 230 | 63 | 210 |
| (5) | 7.8 | 75 | 280 | 70 | 270 | 68 | 270 |

Note: The pH was determined at the beginning and the end of the experiment and did not vary by more than $\pm$ 0.02 units.
The glucose concentration was 0.5% and the temperature was 37°C.

* The pH of the medium was adjusted by either HCl or NaOH.

** The pH of the medium was adjusted by mixing appropriate volumes of monobasic and dibasic potassium phosphate solutions.

*** The pH of the medium was adjusted by either HCl or KOH.

Figure 8: Growth of strains NAB-1 ($^{\Delta}$cya) and NA-1 (cya$^{+}$) on glycerol minimal medium as a function of pH.

Symbols: (A) pH 6.0

(B) pH 7.8

(■) Strain NAB-1 ($^{\Delta}$cya)

(□) Strain NA-1 (cya$^{+}$)

Note: The glycerol concentration was 0.5%.
The Temperature was 37°C.
The pH of the medium was adjusted with either HCl (10% stock solution) or NaOH (2.5 N stock solution).

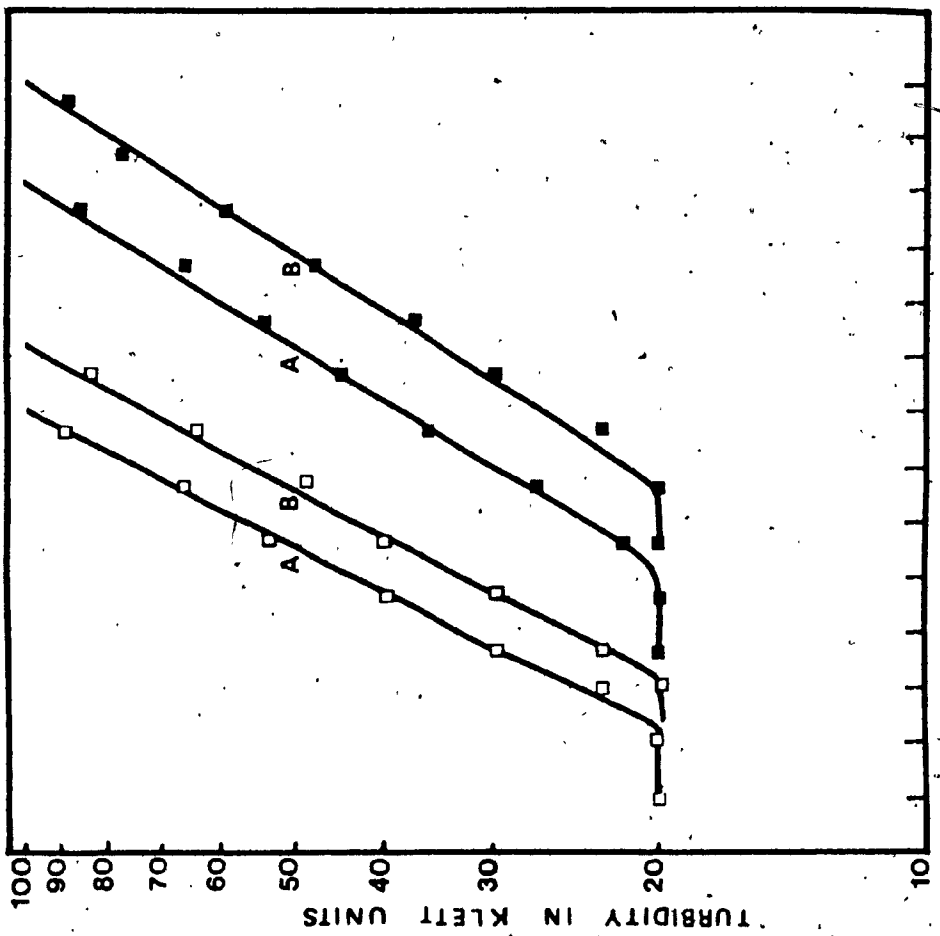

TURBIDITY IN KLETT UNITS
100
90
80
70
60
50
40
30
20
10
A
B
A
B
TIME
in 30 min. intervals

FIGURE 8

Table 7

The effect of the pH of the growth medium on the doubling time of mutant (NAB-1) and parent (NA-1) strains on glycerol and pyruvate minimal medium

| Carbon-source | pH of the medium | Doubling Time (in minutes) in medium adjusted with HCl or NaOH* Strain NA-1 (cya+) | Strain NAB-1 (Δcya) | KOH** Strain NA-1 (cya+) | Strain NAB-1 (Δcya) |
|---|---|---|---|---|---|
| (1) Glycerol | 6.0 | 80 | 90 | 83 | 110 |
| | 7.8 | 80 | 93 | 85 | 113 |
| (2) Pyruvate | 6.0 | 75 | 95 | 78 | 90 |
| | 7.8 | 74 | 220 | 78 | 210 |
| (3) Glucose & Glycerol | 6.0 | 60 | 80 | nd | nd |
| | 7.8 | 60 | 90 | nd | nd |

Note: The glycerol or pyruvate concentrations were 0.5%.
The temperature was 37°C.
* The pH of the medium was adjusted by either HCl or NaOH.
** The pH of the medium was adjusted by either HCl or KOH.
nd Not done.

Figure 9: Growth of strains NAB-1 ($\Delta$cya) and NA-1 (cya$^+$) on pyruvate minimal medium as a function of pH.

Symbols: (A) pH 6.0

(B) pH 7.8

(■) Strain NAB-1 ($\Delta$cya)

(□) Strain NA-1 (cya$^+$)

Note: The pyruvate concentration was 0.5%.
The temperature was 37°C.
The pH of the medium was adjusted with either HCl or NaOH.

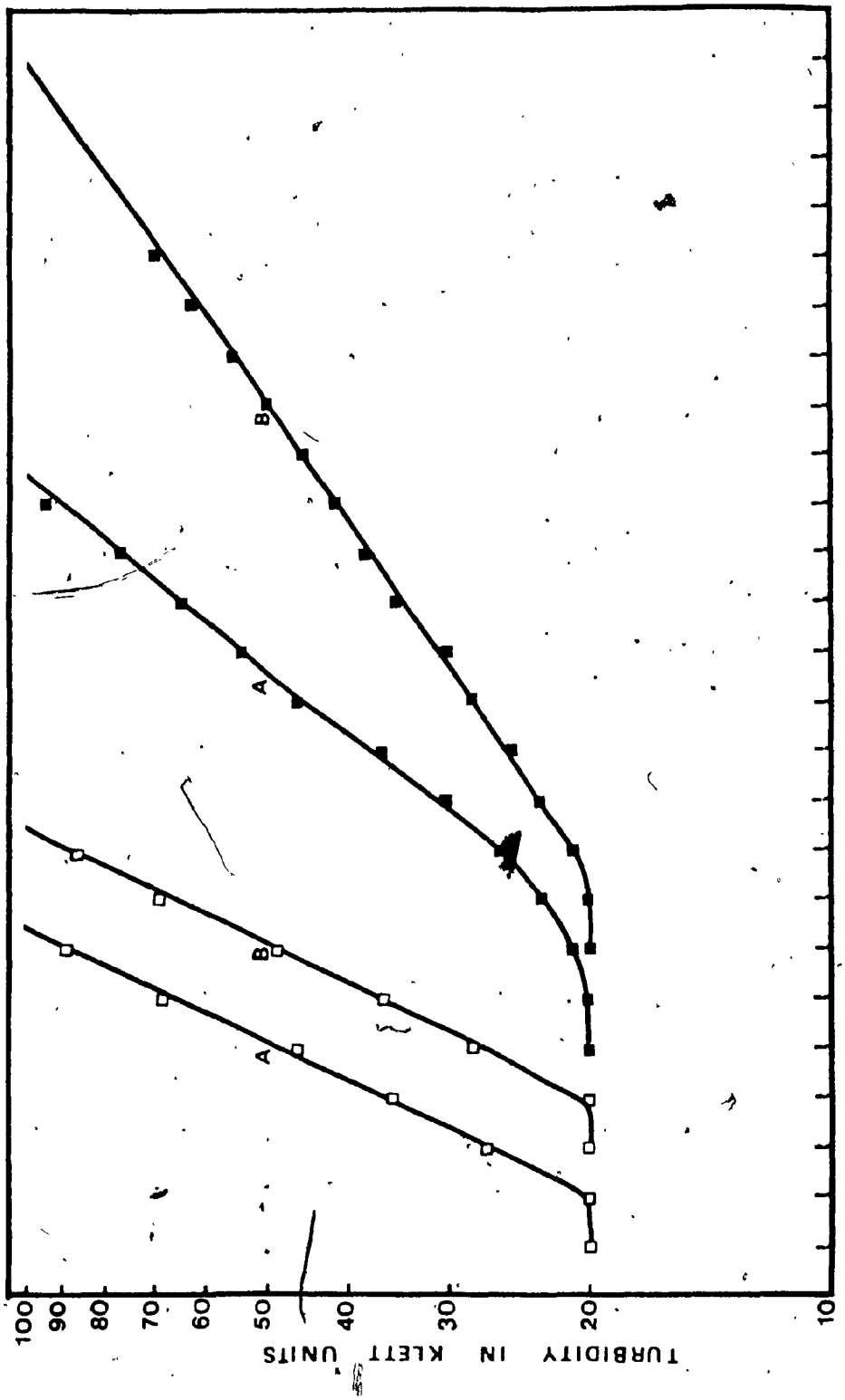

TURBIDITY IN KLETT UNITS
100
90
80
70
60
50
40
30
20
10
A
B
A
B
TIME
in 30 min. intervals

FIGURE 9

(Fig. 9, Table 7).

When glucose and glycerol were given simultaneously as carbon sources, the mutant grew with a doubling time of 80 minutes at pH 6.0, and 90 minutes at pH 7.8, as compared to the 60 minutes for the parent strain at both pH values (Fig. 10, Table 7). Therefore, it seems that the presence of glucose did not inhibit the utilization of glycerol by the mutant strain.

These results indicate that the growth of the mutant, on glucose and pyruvate, is severely affected by the pH of the medium, being faster at lower pHs, while the parent strain grows with almost the same rate at all pHs tested. But, since the growth medium in all these experiments was adjusted with NaOH, it was important to differentiate whether the observation made is in fact due to the increase in pH or due to the increase in the $Na^+$ concentration or both. Therefore, the experiment was repeated using growth medium adjusted with either KOH or with mono-/dibasic potassium phosphate solutions. The results obtained clearly show that the pH of the medium affects the growth rate of the mutant in the same pattern, no matter whether the medium used was adjusted with NaOH or mono-/dibasic salts or KOH (Table 6 and 7). Also, it appears that when NaOH was used to adjust the pH (from 6.4 to 7.0, 7.4 and 7.8) the growth rate of the parent strains, on glucose, decreased slightly more than when KOH or monobasic and dibasic salts were used (Table 6). The growth rate of the mutant did not seem to vary much in any particular fashion but it did seem to be slowest at pHs 7.0 and 7.4 when monobasic and

dibasic salts were used and at pH 7.8 when NaOH was used. On pyruvate the growth rates of the parent as well as the mutant did not appear to be significantly affected either by the addition of NaOH or KOH (Table 7). The parent strain grew slightly more slowly while the mutant strain grew slightly faster when KOH was used to adjust the pH. However, these differences do not appear to be significant enough to alter the conclusion made earlier about the pH-sensitive growth rate of the $\Delta$*cya* mutant.

On glycerol both strains grew slightly more slowly when KOH was used and the growth rates did not seem to be affected by the pH (different from the observations made with glucose and pyruvate).

Figure 10: Growth of strains NAB-1 ($^{\Delta}$cya) and NA-1 (cya$^{+}$) on medium, supplemented with glucose and glycerol, as a function of pH.

Symbols: (A) pH 6.0

(B) pH 7.8

(■) Strain NAB-1 ($^{\Delta}$cya)

(□) Strain NA-1 (cya$^{+}$)

Note: The glycerol and glucose concentrations were 0.5%. The temperature was 37°C.

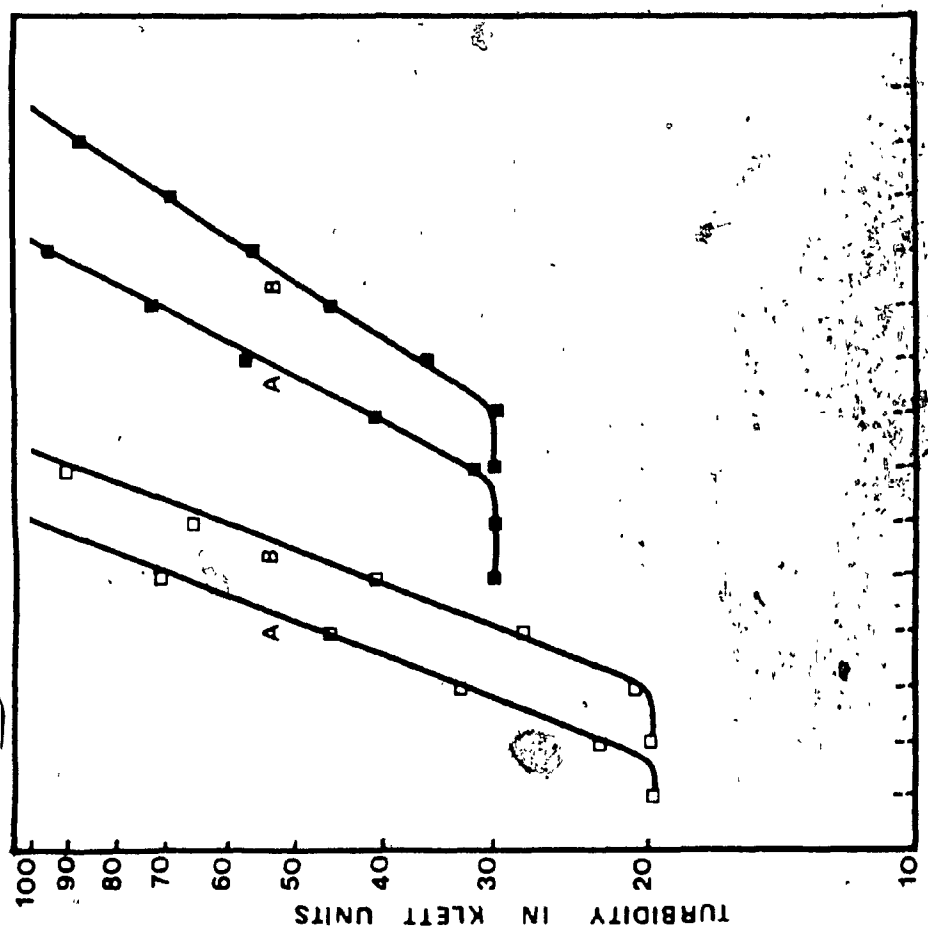

TURBIDITY IN KLETT UNITS
100
90
80
70
60
50
40
30
20
10
A
B
A
B
TIME
in 30 min. intervals

FIGURE 10

## VI. Effect Of pH On Yield Of Cell Material In Glucose And Glycerol Minimal Medium:

Since the mutant grew more slowly on glucose than the parent and the growth was also affected by pH, whereas on glycerol its growth was only slightly slower than the parent and was not affected by pH, the efficiency with which the mutant uses glucose and glycerol under various pH conditions was compared. This was done by comparing the amount of cell material made by each strain from limiting amounts of carbon source at various pHs.

Yields were determined in batch cultures at two limiting concentrations of carbon source (0.04% and 0.08%) and at pH values of the medium ranging from 6.0 to 7.8. On glucose minimal medium at pH 6.0, the mutant gave 20% less cell mass than that of the parent strain and at pH 7.8 the mutant gave approximately 60% less cell mass than the parent strain (Table 8). At all pHs and in both strains, yields were higher when the medium pH was adjusted with KOH rather than with NaOH (Table 8).

Although on glycerol minimal medium the growth rate of the mutant strain was not very much different from the parent and was not altered at high pH, the yield was lower than that of the parent strain and was also affected by the pH of the medium (Table 9). As was seen with glucose, at all pHs, the growth yields were higher in cultures grown on medium adjusted by KOH than those adjusted by NaOH (Table 9).

Therefore, the mutant seems to be less efficient at all pHs but the deficiency was greater as the pH increased. This was true for

Table 8

The effect of external pH
on the yield of cell material in glucose grown cultures

| Concentration of glucose in the medium | Strain | pH of the Medium | Protein in the culture (μg/ml) NaOH* | | KOH** | |
|---|---|---|---|---|---|---|
| | | | Exp. I | Exp. II | Exp. I | Exp. II |
| (1) 0.04% | NA-1 ($cya^+$) | 6.0 | 96 | 110 | 160 | 160 |
| | | 6.4 | 120 | 113 | 160 | 150 |
| | | 7.0 | 111 | 110 | 160 | 150 |
| | | 7.4 | 119 | 108 | 140 | 150 |
| | | 7.8 | 109 | 112 | 150 | 140 |
| | NAB-1 ($\Delta cya$) | 6.0 | 88 | 83 | 140 | 130 |
| | | 6.4 | 84 | 78 | 120 | 110 |
| | | 7.0 | 73 | 70 | 120 | 80 |
| | | 7.4 | 68 | 59 | 110 | 70 |
| | | 7.8 | 54 | 45 | 90 | 60 |
| (2) 0.08% | NA-1 ($cya^+$) | 6.0 | 210 | 217 | 260 | 300 |
| | | 6.4 | 215 | 217 | 270 | 320 |
| | | 7.0 | 225 | 222 | 280 | 310 |
| | | 7.4 | 220 | 202 | 230 | 310 |
| | | 7.8 | 210 | 214 | 230 | 300 |
| | NAB-1 ($\Delta cya$) | 6.0 | 173 | 171 | 240 | 250 |
| | | 6.4 | 135 | 159 | 220 | 210 |
| | | 7.0 | 125 | 130 | 190 | 200 |
| | | 7.4 | 108 | 110 | 130 | 150 |
| | | 7.8 | 109 | 97 | 90 | 120 |

Note: Each result is an average of three determinations.
The temperature was 37°C.
* The pH of the medium was adjusted with NaOH.
** The pH of the medium was adjusted with KOH.

Table 9

The effect of external pH on the yield of cell material with limiting glycerol concentrations

| Concentration of glycerol in the medium | Strain | pH of the Medium | Protein in the culture (µg/ml) NaOH* | | KOH** | |
|---|---|---|---|---|---|---|
| | | | Exp. I | Exp. II | Exp. I | Exp. II |
| (1) 0.04% | NA-1 | 6.0 | 120 | 123 | 120 | 140 |
| | ($cya^+$) | 7.0 | 123 | 120 | 140 | 120 |
| | | 7.8 | 122 | 124 | 130 | 130 |
| | NAB-1 | 6.0 | 94 | 101 | 110 | 110 |
| | ($\Delta$cya) | 7.0 | 50 | 61 | 100 | 90 |
| | | 7.8 | 37 | 49 | 80 | 70 |
| (2) 0.08% | NA-1 | 6.0 | 240 | 227 | 270 | 270 |
| | ($cya^+$) | 7.0 | 246 | 210 | 310 | 270 |
| | | 7.8 | 238 | 216 | 300 | 260 |
| | NAB-1 | 6.0 | 156 | 188 | 240 | 230 |
| | ($\Delta$cya) | 7.0 | 96 | 111 | 210 | 180 |
| | | 7.8 | 82 | 98 | 130 | 130 |

Note: Each result is an average of three determinations.
The temperature was 37°C.
* The pH of the medium was adjusted with NaOH.
** The pH of the medium was adjusted with KOH.

both glycerol and glucose grown cells. That is, glucose grown cells showed a deficiency in both growth rate and yield, and glycerol grown in yield only.

VII. Effect Of Exogenous cAMP On The Growth Rate In Glucose Minimal Medium At pH 7.8:

In the preceding sections, the growth of the mutant was seen to be affected by the pH of the medium. In section IV, it has been shown that at pH 6.4 the slow growth of the mutant can be restored to normal by the addition of cAMP. If the greater decrease in growth at pH 7.8 than at pH 6.4 was due to a deficiency of cAMP (as it was at pH 6.4) then the addition of exogenous cAMP should be able to restore it to normal as it did at pH 6.4. Therefore, growth rates at pH 7.8, were determined using various concentrations of exogenous cAMP.

At pH 7.8, the doubling time of the mutant was approximately 243 minutes as compared to 67 minutes for the parent strain (Table 10). The addition of cAMP increased the growth rate of the mutant but did not have much effect in the parent strain. At a concentration of $1.0 \times 10^{-4}$ M, exogenous cAMP reduced the doubling time of the mutant to 120 minutes. At the highest cAMP concentration used, $5.0 \times 10^{-3}$ M, the mutant grew fastest (doubling time being 75 minutes), to a near normal value, while the growth of the parent strain was slightly inhibited.

Therefore, it seems that the inhibition of growth of the mutant at pH 7.8 was also due to a deficiency of cAMP (as was observed at pH 6.4), since the addition of exogenous cAMP at a concentration of $5.0 \times 10^{-3}$ M brought the growth rate of the mutant to a near normal value. This concentration is higher than the concentration needed to restore a normal growth of the mutant at pH 6.4.

Table 10

The effect of various concentrations of exogenous cAMP on the doubling time on glucose of parent (NA-1) and mutant (NAB-1) strains at pH 7.8

| | Concentration of cAMP in the medium (M) | Doubling Time (in minutes) Strain NA-1 ($cya^+$) | Strain NAB-1 ($\Delta cya$) |
|---|---|---|---|
| (1) | 0 | 67 | 243 |
| (2) | $1.0 \times 10^{-4}$ | 62 | 120 |
| (3) | $2.5 \times 10^{-4}$ | 65 | 92 |
| (4) | $5.0 \times 10^{-4}$ | 63 | 88 |
| (5) | $1.0 \times 10^{-3}$ | 68 | 83 |
| (6) | $2.5 \times 10^{-3}$ | 68 | 80 |
| (7) | $5.0 \times 10^{-3}$ | 71 | 75 |

Note: The glucose concentration was 0.5%.
The experimental temperature was 37°C and the pH of the medium was adjusted with KOH.

VIII. Effect Of pH On The Growth Rate And The Cell Yield Of A Few Other cya Mutants In Glucose Minimal Medium:

In the preceding sections, the growth of a $^{\Delta}$cya mutant was seen to be highly sensitive to the high pH values of the medium. If this pH-sensitivity is a general characteristic of the cya mutation, one might then expect to see it in other cya mutants with different genetic backgrounds. Therefore, the effect of pH on the growth properties of two other well known $^{\Delta}$cya mutants (81, 108) were studied. Indeed the growth rate and the cell yield per unit carbon source were both found to be pH-sensitive (Table 11) as was seen in case of the strain NAB-1.

AT pH 6.0 the doubling times of the mutant strains, CA8306 and CA8303, were 110 and 100 minutes respectively, while that of the parent strain was 65 minutes. The doubling time of the parent strain was not affected by the pH of the medium, while that of the two mutant strains increased gradually from 126 minutes at pH 7.0 to 153 and 173 minutes, respectively, for strains CA8306 and CA8303.

The two mutant strains produced less cell mass per unit carbon source as the external pH increased. The yields were lower under all pH conditions than that of the parent strain (Table 12) except for the strain CA8303 at pH 6.0 where the cell yield was nearly the same as that of the parent strain. However, in all cases the pH-sensitivity of the cell yield was well pronounced.

These results clearly show that the pH-sensitivity of the growth is a general characteristic of the cya mutants, since it was seen in strains with different genetic backgrounds.

Table 11

The effect of pH of the growth medium on the doubling time of $\Delta$cya mutants, CA8306 and CA8303, and their parent, CA8001, on glucose minimal medium

| pH of the medium | Doubling Time (in minutes) Strain CA8001 (cya$^+$) | Strain CA8306 ($\Delta$cya) | Strain CA8303 ($\Delta$cya) |
|---|---|---|---|
| (1) 6.0 | 65 | 110 | 100 |
| (2) 7.0 | 65 | 126 | 126 |
| (3) 7.8 | 65 | 153 | 173 |

Note: The glucose concentration was 0.5% and the temperature was 37°C. The pH of the medium was adjusted by either HCl or KOH. The medium contained thiamine (1 µg/ml) and casamino acid (0.1%)

Table 12

The effect of external pH
on the yield of cell material in glucose grown cultures

| Concentration of glucose in the medium | | Strain | pH of the Medium | Protein in the culture ( µg/ml) Exp. I | Exp. II | Exp. III |
|---|---|---|---|---|---|---|
| (1) | 0.04% | CA8001 | 6.0 | 120 | 120 | 110 |
| | | ($cya^+$) | 7.0 | 125 | 115 | 110 |
| | | | 7.8 | 115 | 125 | 115 |
| | | CA8306 | 6.0 | nd | 115 | 90 |
| | | ($\Delta$cya) | 7.0 | nd | 110 | 85 |
| | | | 7.8 | nd | 100 | 70 |
| | | CA8303 | 6.0 | 120 | 115 | 110 |
| | | ($\Delta$cya) | 7.0 | 80 | 110 | 95 |
| | | | 7.8 | 70 | 90 | 75 |
| (2) | 0.08% | CA8001 | 6.0 | 230 | 200 | 230 |
| | | ($cya^+$) | 7.0 | 240 | 190 | 230 |
| | | | 7.8 | 200 | 200 | 220 |
| | | CA8306 | 6.0 | nd | 180 | 160 |
| | | ($\Delta$cya) | 7.0 | nd | 170 | 140 |
| | | | 7.8 | nd | 140 | 120 |
| | | CA8303 | 6.0 | 180 | 240 | 210 |
| | | ($\Delta$cya) | 7.0 | 140 | 180 | 155 |
| | | | 7.8 | 120 | 105 | 110 |

Note: Each result is an average of three determinations.
The temperature was 37°C.
The pH of the medium was adjusted with KOH.
The medium contained thiamine (1 µg/ml) and proline (50 µg/ml).

## IX. Effect Of Shifting The pH Of The Growth Medium On The Growth Rate In The Presence And Absence Of Exogenous cAMP:

In the preceding experiments, the mutant's growth rate was seen to be greatly decreased at high pH values. These experiments involved different cultures each grown at a particular pH. One might then expect the cya mutants to have considerable difficulty in adapting to a sudden increase in pH. To test this, the cells were grown at pH 6.0 and NaOH was added suddenly to bring the culture to pH 7.8. The reverse experiment, growing cells at pH 7.8 and adding HCl, was also carried out. Both pH shifts resulted in the mutant ceasing to grow for a period of 40-60 minutes (Fig. 11, 12). After the shift, the mutant resumed growth at the rate characteristic of the new pH. The growth of the parent strain was not noticeably affected by either shift.

Since growth was faster and more efficient at low pH, one might expect the transition from high pH to low to be easier than the reverse shift, because a change from high pH to low should be able to relax the strain (whatever it be) of high pH almost immediately. The shift from high to low pH was easier than the reverse shift, as judged by the lag period being approximately 40 minutes in the former case and 60 in the latter. However, a pronounced lag was seen on shifting the pH downward. It seemed possible that the lag could be caused by something other than pH. However, control experiments done by adding minimal medium and a solution of sodium chloride showed that the lag in the growth was not due to dilution of the culture, the method used

Figure 11: Growth of strains NA-1 ($cya^+$) and NAB-1 ($\Delta cya$) after shift of external pH from 6.0 to 7.8.

Symbols: (A) pH 6.0

(B) pH 6.0 → 7.8

(C) pH 6.0 → 7.8 with cAMP addition at the time of the shift.

(○) Strain NA-1 ($cya^+$)

(●) Strain NAB-1 ($\Delta cya$)

Note: 0.5% glucose was the growth substrate.
The temperature was 37°C.
↓indicates time of pH shift and cAMP addition.
The cAMP concentration used was $2.5 \times 10^{-3}$ M.
The pH was shifted by adding 1.2 ml of NaOH (2.5 N stock solution) to 20 ml of the culture.

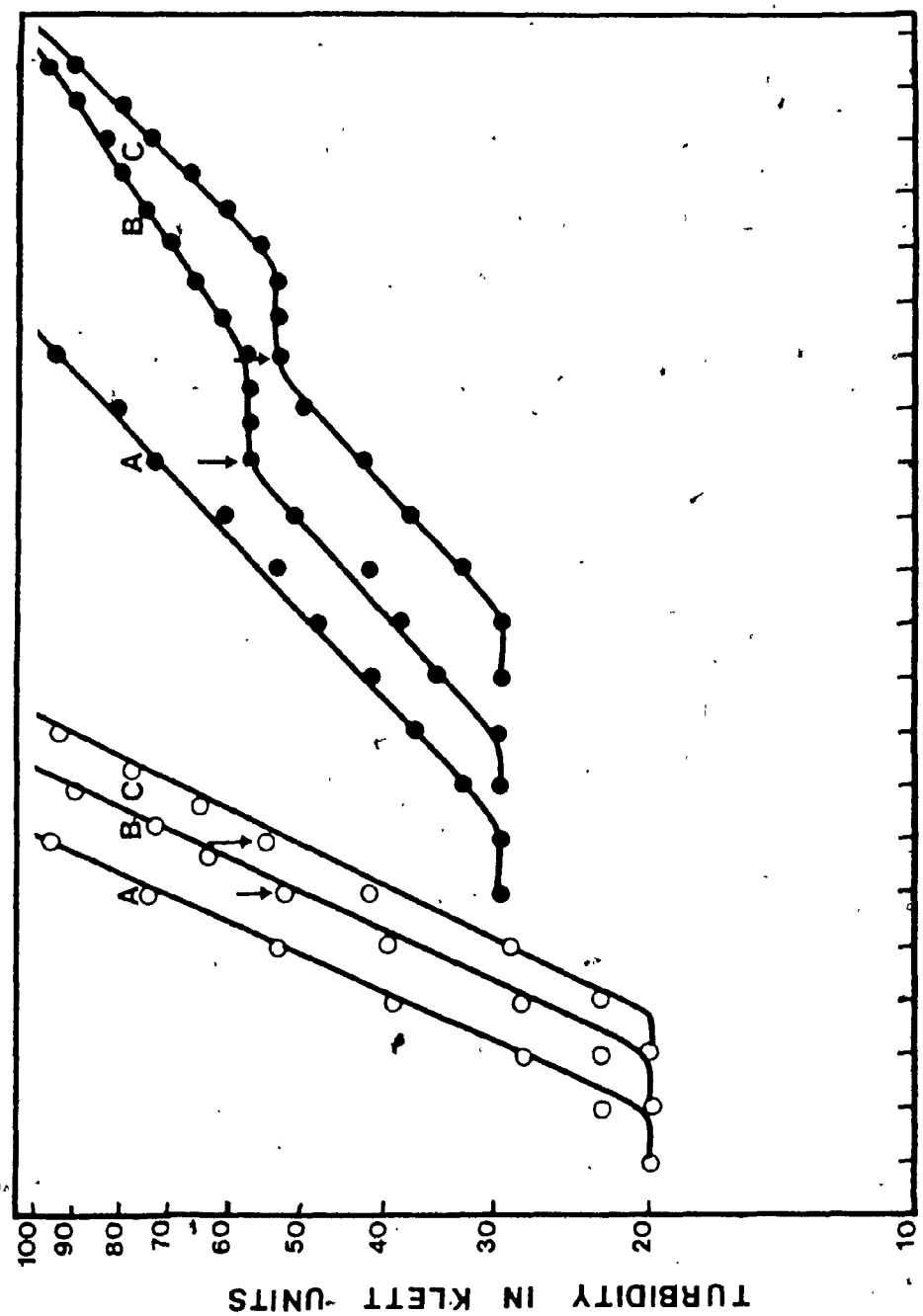

TURBIDITY IN KLETT -UNITS
100
90
80
70
60
50
40
30
20
10
A
B
C
A
B
C
TIME
in 30 min. intervals

FIGURE 11

Figure 12: Growth of strains NA-1 ($cya^+$) and NAB-1 ($\Delta cya$) after shift of external pH from 7.8 to 6.0.

Symbols: (A) pH 6.0

(B) pH 7.8 → 6.0

(C) pH 7.8 → 6.0 with cAMP addition at the time of the shift.

(○) Strain NA-1 ($cya^+$)

(●) Strain NAB-1 ($\Delta cya$)

Note: 0.5% glucose was the growth substrate.
The temperature was 37°C.
↓ indicates time of pH shift and cAMP addition.
The cAMP concentration was $2.5 \times 10^{-3}$ M.
The pH was shifted by adding 0.7 ml of HCl (10% stock solution) to 20 ml of the culture.

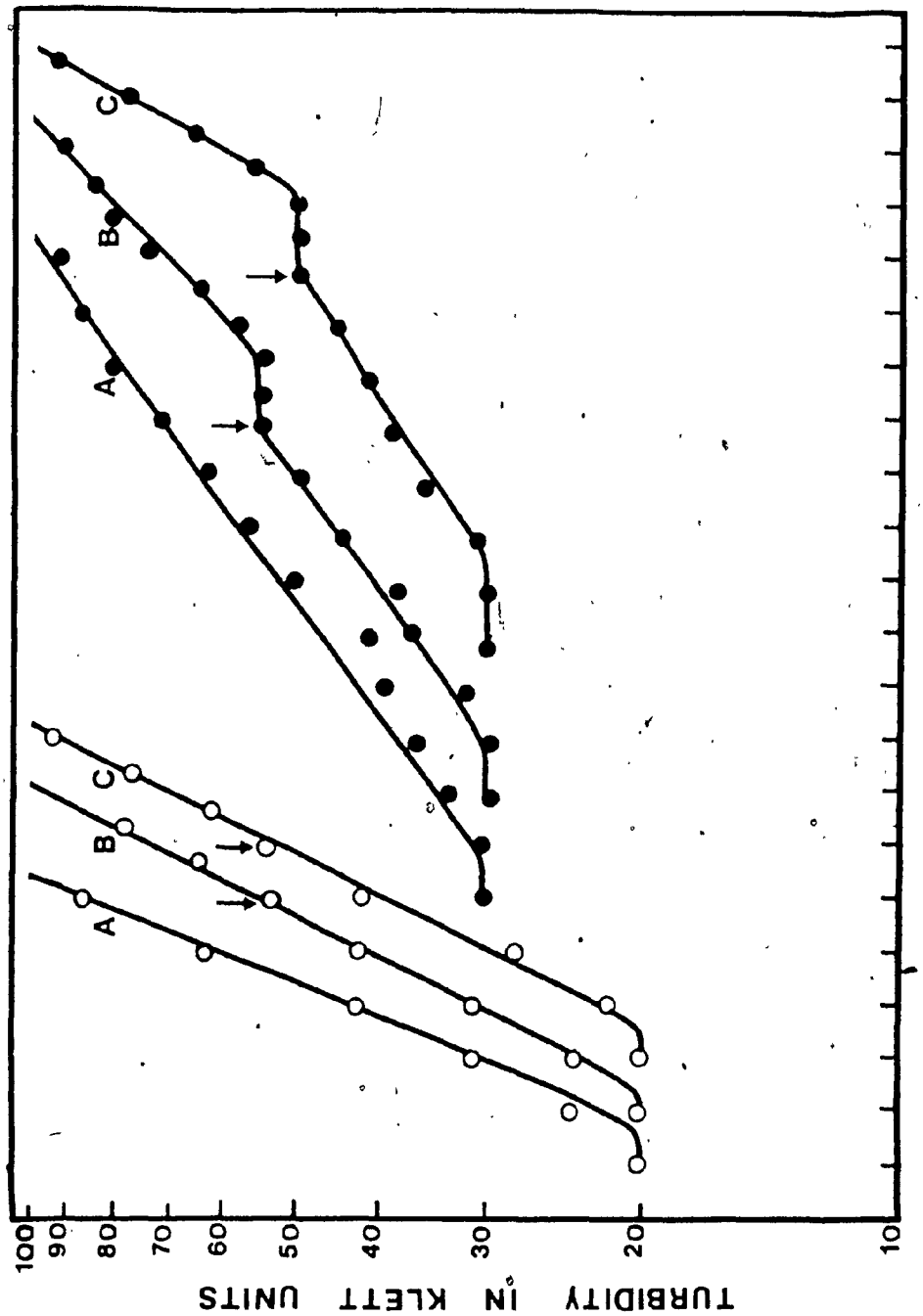

TURBIDITY IN KLETT UNITS
100
90
80
70
60
50
40
30
20
10
A
B
C
A
B
C
TIME
in 30 min. intervals

FIGURE 12

for shifting the pH, the addition of chloride or sodium ions to the medium (Fig. 13).

In any case one might expect that the addition of cAMP should influence the lag period. Addition of cAMP at the time of shift did not affect the lag period observed but did increase the growth rate later on (Fig. 11, 12). Therefore, the presence of cAMP at the time of the pH-shift is not sufficient to enable the strain to adapt to the pH change. This adaptation then, depends on the function of some structure, synthesis (and/or regulation of synthesis) of which only occurs in the presence of cAMP.

Figure 13: Effect of the addition of growth medium and sodium chloride on the growth of strains NA-1 ($cya^+$) and NAB-1 ($\Delta cya$).

Symbols: (A) pH 6.0, 1.2 ml medium* added

(B) pH 7.8, 1.2 ml medium* added

(C) pH 6.0, 0.7 ml 10% NaCl** added

(D) pH 7.8, 0.7 ml 10% NaCl** added

(▼) Strain NA-1 ($cya^+$)

(●) Strain NAB-1 ($\Delta cya$)

Note: 0.5% glucose was the growth substrate.
The temperature was 37°C.
* 1.2 ml of medium equals to the volume of HCl added to shift the pH from 7.8 to 6.0.
** 0.7 ml of NaCl equals the amount of NaOH added to shift the pH from 6.0 to 7.8.

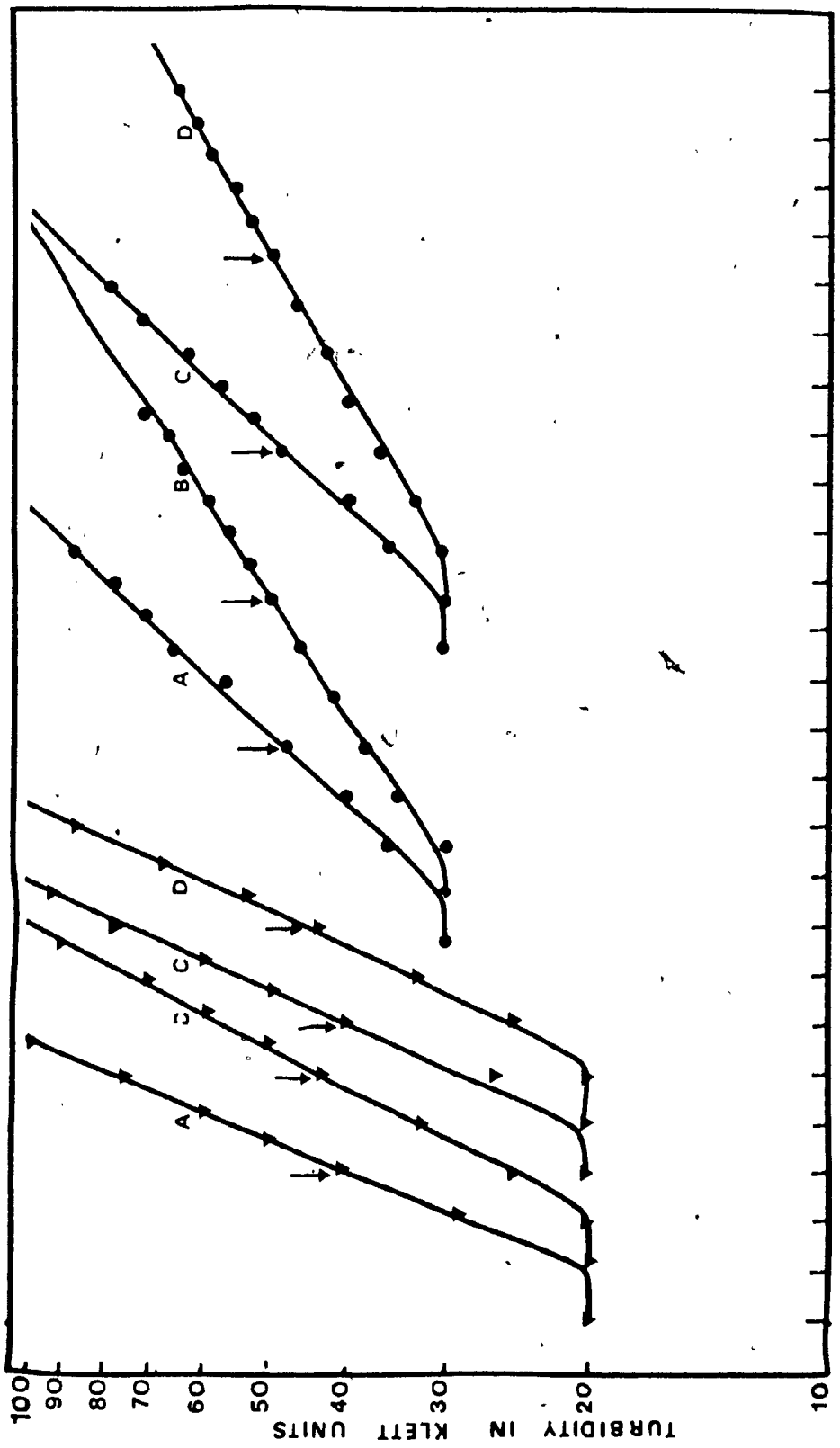

TURBIDITY IN KLETT UNITS
100
90
80
70
60
50
40
30
20
10
A
B
C
D
TIME in 30 min. intervals

FIGURE 13

## X. Involvement Of The cAMP-CRP Complex In The Mechanism Of pH-Sensitivity Of The Adenyl Cyclase Mutant:

The experiments described in previous sections indicate a role of cAMP in cell function at high pH. The mechanism by which cAMP mediates catabolite repression is understood in considerable detail. The model involves a cAMP receptor protein (CRP) that acquires an active conformation after binding to cAMP and in its active form binds to the particular DNA sequences (promotor region) initiating transcription (68, 153, 155, 157). If the role of cAMP in the pH-sensitivity follows the same model, CRP must also be involved. Therefore, a study of growth characteristics of a well known $\Delta$*crp* mutant was made. The growth rate of the $\Delta$*crp* mutant (EC8439) was found to be pH-sensitive, decreasing gradually as the pH of the medium increased (Table 13). The doubling times at pH 6.0, 7.0 an 7.8 were 95, 125 and 160 minutes, respectively. The yield of cell material was also affected by the pH of the medium in the same pattern as that of the growth rate. At pH 6.0, 7.0 and 7.8, the yields were 100, 81 and 69 µg of protein per ml of culture respectively, when 0.04% of glucose was present in the culture. At a concentration of 0.08%, the yields were 149, 117 and 94 µg protein per ml of culture at pH 6.0, 7.0 and 7.8 respectively (Table 13).

These results indicate that mutation in the *crp* gene also renders the bacteria pH-sensitive as did the mutation in the *cya* gene.

Since, in the above mentioned study, the parent strain of the $\Delta$*crp* mutant was not available for comparison, the $\Delta$*crp* mutation was

Table 13

The effect of external pH on the growth rate and yields of cell material in glucose grown cells of strain EC8439 ($\Delta$ crp)

| pH of the medium | Doubling Time (in minutes)* | Protein in the culture (µg/ml) Glucose 0.04% | Glucose 0.08% |
|---|---|---|---|
| (1) 6.0 | 95 | 100 | 149 |
| (2) 7.0 | 125 | 81 | 117 |
| (3) 7.8 | 160 | 69 | 94 |

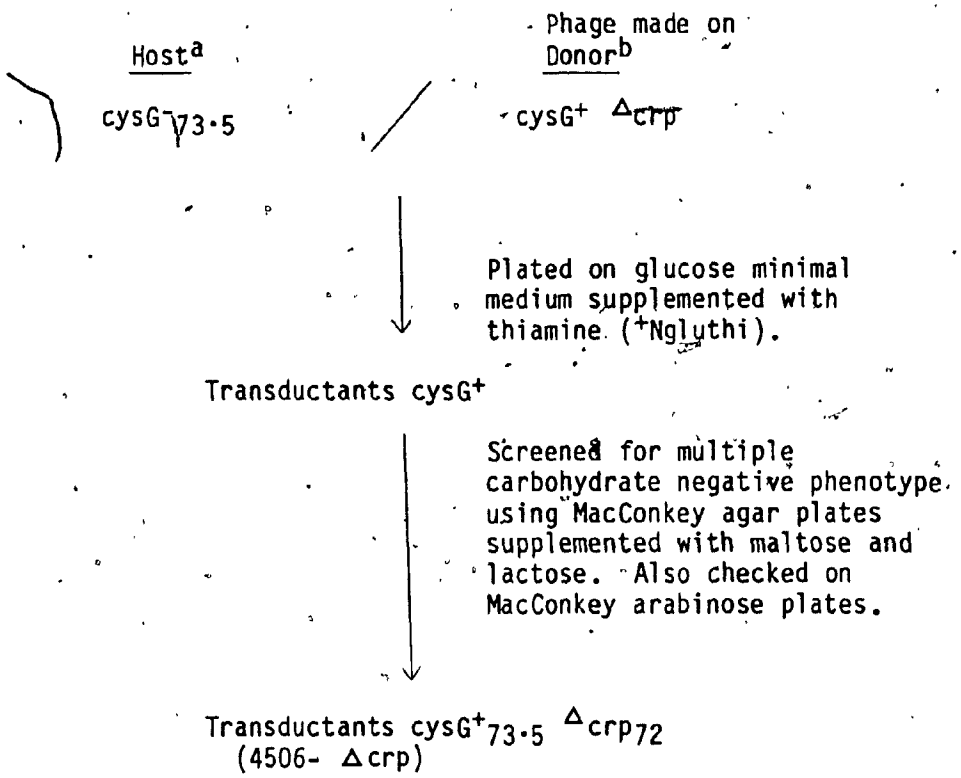
Note: The temperature was 37°C.
The pH of the medium was adjusted by either HCl or KOH.
The medium contained thiamine (1 µg/ml).
*The glucose concentration was 0.5% and the medium was supplemented with 0.1% casamino acid.

transduced, with linkage to cysG, from the strain EC8439 to the strain 4506. The scheme of the transduction is given in Figure 14. Phage P1CM was grown on the donor, strain EC8439. The recipient strain, 4506, carried a cysG mutation and was therefore a cysteine requiring strain. The selection was made for cysteine independent transductants by plating on minimal medium supplemented with glucose and thiamine. The cysteine independent transductants were screened for the $^{\Delta}$crp mutation by testing on MacConkey plates supplemented with lactose and maltose, with and without cAMP. The possible $^{\Delta}$crp transductants were also tested on MacConkey arabinose plates and on lactose minimal medium, in the presence and absence of cAMP. Transductants that were pleiotropic carbohydrate negative under all the above mentioned conditions were considered to be $^{\Delta}$crp strains.

Of 180 cystein independent transductants 17 were $^{\Delta}$crp. One of these 17 $^{\Delta}$crp strains (4506-$\Delta$crp) and one of the cysteine independent crp$^{+}$ strain (4506-W) were further studied for their growth characteristics at pH 6.0 and 7.8. The growth rate of this $^{\Delta}$crp mutant was slow and pH-sensitive, the doubling time being 138 and 225 minutes at pH 6.0 and 7.8, respectively (Table 14). The parent strain grew with a doubling time of 78 minutes at pH 6.0 and was not significantly affected by the pH of the medium. The yield of protein per unit carbon source also decreased at high pH values (Table 14).

Two other strains carrying deleted cya as well as crp genes were also studied for their growth characteristics. These double deletion mutants were found to be pH-sensitive. The growth rates of the

Figure 14. Scheme for transduction of $\Delta$crp mutation from strain EC8439 ( $\Delta$crp) to strain 4506 (cysG$^-$).




Further tested on MacConkey maltose-lactose plates supplemented with cAMP, and on minimal medium with lactose and thiamine.

[a]Host strain used is 4506
[b]Donor strain used is EC8439

Note: The numbers after the gene symbols indicate the map locations.

Table 14

The effect of external pH on the growth rate and yield of cell material in glucose grown cells

| Strains | pH of the medium | Doubling Time (in minutes)* | Protein in the culture (µg/ml) Glucose 0.04% | Glucose 0.08% |
|---|---|---|---|---|
| (1) 4506-W ($crp^+$) | 6.0 | 78 | 111 | 188 |
| | 7.8 | 80 | 106 | 177 |
| (2) 4506-$\Delta$crp ($\Delta$crp) | 6.0 | 138 | 86 | 153 |
| | 7.8 | 225 | 75 | 127 |
| (3) CA8001 ($cya^+$) | 6.0 | 65 | 116 | 220 |
| | 7.0 | 65 | 117 | 220 |
| | 7.8 | 65 | 118 | 208 |
| (4) CA8496 ($\Delta$cya $\Delta$crp) | 6.0 | 126 | 105 | 225 |
| | 7.0 | 152 | 97 | 165 |
| | 7.8 | 187 | 77 | 135 |
| (5) CA8445 ($\Delta$cya $\Delta$crp) | 6.0 | 125 | nd | nd |
| | 7.0 | 163 | nd | nd |
| | 7.8 | 203 | nd | nd |

Note: The temperature was 37°C.
The pH of the medium was adjusted by either HCl or KOH.
The medium contained thiamine (1 µg/ml).
*The glucose concentration was 0.5% and the medium was supplemented with 0.1% casamino acid.

strains CA8496 and CA8445 were 126 and 125 minutes, respectively, at pH 6.0 as compared to 65 minutes for that of the parent strain (Table 14). In both strains the doubling time decreased further at pH 7.0 and 7.8. Similarly, the growth yields also were pH-sensitive in both the double deletion mutants (Table 14) as would be expected if both cAMP and CRP play a role in developing resistance to the external pH.

## XI. Genetic Evidence Of The Involvement Of cAMP-CRP Complex In The Mechanism Of pH-Sensitivity Of The Adenyl Cyclase Mutant:

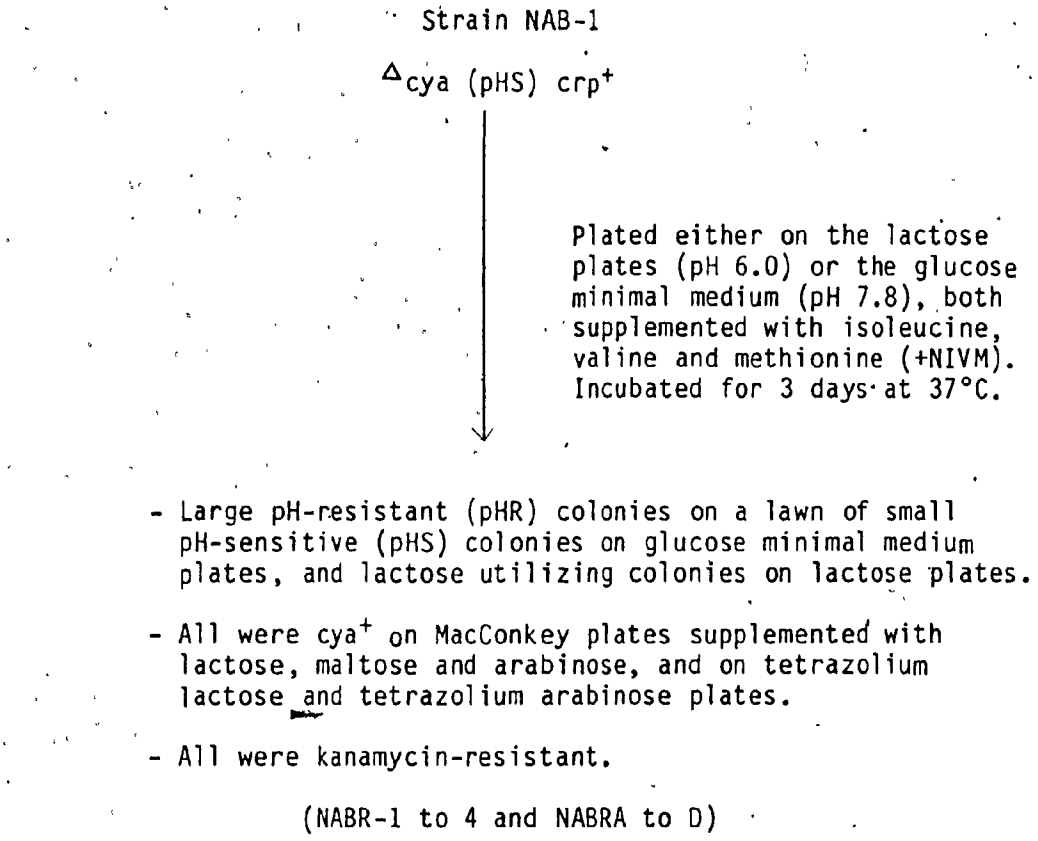
The study of growth characteristics described in the preceding sections showed that cAMP/CRP complex might be involved in the mechanism of pH-resistance, since the mutation in *cya* or *crp* (or both) results in a slow and pH-sensitive growth. In the case of the *cya* mutants, this impaired growth rate returned to normal when exogenous cAMP was provided. Further evidence for this involvement was sought by the application of genetic techniques as have been extensively applied for the study of the catabolite sensitive genes.

Since it is possible to obtain lactose fermenting mutants by mutations that change CRP (20, 37, 197), then it should also be possible to get a pH-resistant (pHR) $^{\Delta}$*cya* mutant in which cAMP-receptor protein is independent of cAMP for its activation. The following set of experiments describe the selection procedure for pHR mutants and provide the genetic evidence that this mutation is in the *crp* gene that codes for the cAMP-receptor protein.

### A. Selection for pH resistant (pHR) mutants of $^{\Delta}$*cya* strain:

The growth of the $^{\Delta}$*cya* mutant on glucose minimal medium was very slow at pH 7.8. This property was used as a tool for selecting fast growing pHR mutants. For this purpose, strain NAB-1 was plated either on the glucose minimal medium at pH 7.8 or on the lactose minimal medium, and after three days of incubation the fast growing (large) colonies were isolated from glucose plates and the lactose utilizing colonies from lactose plates (Fig. 15). Eight independently

Figure 15. Scheme for selection of pH-resistant derivatives from an adenyl cyclase deletion mutant ( $^{\Delta}$cya).



isolated pH resistant (pHR) colonies were isolated and purified, four from each kind of plates. These were also tested for their ability to produce acid on MacConkey plates, supplemented with lactose, maltose and arabinose, and were all found to be positive. All these mutants were kanamycin resistant, and therefore still carried the element $Tn5^{kan}R$. These mutants could not be $cya^+$ revertants since the *cya* mutation was a deletion mutation (26).

The growth rate of all these eight independently isolated pH-resistant $^{\Delta}$*cya* mutants (NABR-1 to 4 and NABR-A to D) were studied at two pHs with and without exogenous cAMP (Table 15). The doubling time of all these mutants varied from 80 to 90 minutes at pH 6.0 and from 90 to 100 minutes at pH 7.8. These values are slightly higher than the normal values (63 minutes at pH 6.0 and 68 minutes at pH 7.8). Addition of cAMP at a concentration of $2.5 \times 10^{-3}$ M appears to have slightly decreased the doubling time of the normal ($cya^+$) strain. But the pH-resistant $^{\Delta}$*cya* mutants showed no significant effect after the addition of cAMP (Table 15).

B. Genetic evidence that the strains NABR-1 to 4 And NABR-A to D (pH-resistant $^{\Delta}$*cya*) carry mutations in their *crp* genes

Resistance to the external pH (pHR) may occur in many ways. To test whether the pHR $^{\Delta}$*cya* strains described in the previous section in fact carried mutations in their *crp* genes, a genetic approach was used. The method depends on having a $^{\Delta}$*cya* recipient strain that also carries a mutation near the *crp* gene, in this case $cysG_{73.5}$. A functional *cysG* gene is then transduced from the eight pHR $^{\Delta}$*cya*

Table 15

The effect of pH on the growth rate of the pH-resistant $\Delta$*cya* mutants in the presence or absence of exogenous cAMP

| Strains | pH of the medium | Doubling Time (in minute) without cAMP | Doubling Time (in minute) with cAMP ($2.5 \times 10^{-3}$ M) |
|---|---|---|---|
| NA-1 | 6.0 | 63 | 60 |
| | 7.8 | 68 | 63 |
| NABR-1 | 6.0 | 90 | 83 |
| | 7.8 | 95 | 85 |
| NABR-2 | 6.0 | 83 | 80 |
| | 7.8 | 100 | 88 |
| NABR-3 | 6.0 | 85 | 80 |
| | 7.8 | 100 | 88 |
| NABR-4 | 6.0 | 83 | 83 |
| | 7.8 | 93 | 88 |
| NABR-A | 6.0 | 83 | 80 |
| | 7.8 | 96 | 90 |
| NABR-B | 6.0 | 83 | 78 |
| | 7.8 | 95 | 90 |
| NABR-C | 6.0 | 83 | 85 |
| | 7.0 | 90 | 90 |
| NABR-D | 6.0 | 80 | 80 |
| | 7.8 | 95 | 90 |

Note: The glucose concentration was 0.5%.
The temperature was 37°C.
The pH of the medium was adjusted with KOH or HCl.
NA-1 is a $cya^+$ strain.
NABR-1 to 4 and NABR-A to D are pH-resistant $\Delta$*cya* strains.

strains to the $^{\Delta}$cya cysG$^{-}$ recipient with selection for cysteine-independent transductants. Since crp is near cysG, some of the transductants will also carry the crp allele from the pHR strain. If pH-resistance is due to a change in crp, these strains should be pH-resistant and sugar fermenting. To test this, the following set of experiments was performed.

- Construction of a cysG $^{\Delta}$cya strain:

The $^{\Delta}$cya mutation was transferred into the strain 4506 carrying cysG mutation, by the same method that was used in section I. The scheme of the experiment is illustrated in Figure 16. Of 250 kanamycin resistant transductants screened, seven (4506Δ-1 to 7) proved to be $^{\Delta}$cya (slow growing, pH-sensitive and pleiotropic carbohydrate non-utilizing).

- Cotransduction of crp* allele into strain 4506-Δ1 (cysG $^{\Delta}$cya) as an unselected marker with the cysG$^{+}$ as a selected marker

This experiment was done to determine if the pH-resistant $^{\Delta}$cya strains (NABR-1 to 4 and NABR-A to D) did carry a mutation at their crp loci that gave them cya$^{+}$ phenotype. Strain 4506-Δ1 that carries cysG and $^{\Delta}$cya mutations was used as recipient, and pH-resistant $^{\Delta}$cya crp* strains were used as donors (Fig. 17). Cystein-independent (cysG$^{+}$) transductants were selected and screened further to select any which might have a cya$^{+}$ phenotype, as expected if pH-resistance were caused by a change at the crp locus. Indeed, in each of these eight

Figure 16. Scheme for transduction of $^{\Delta}$cya mutation from strain JB-1 ($^{\Delta}$cya) to strain 4506 (cysG$^-$).

| Host[a] | Phage made on Donor[b] |
|---|---|
| cysG$^-_{73\cdot5}$ cya$^+_{83}$ KanS | cysG$^+$ $^{\Delta}$cya$_{83}$ ilv::Tn5$_{84}$ KanR |

↓ Plated on rich medium (LB) supplemented with kanamycin.

Transductants KanR (ilv::Tn5)

↓ Screened for multiple carbohydrate negative phenotype using MacConkey agar plates supplemented with maltose, lactose and arabinose. Also checked on tetrazolium lactose and tetrazolium arabinose plates.

Transductants KanR (ilv::Tn5)$_{84}$ $^{\Delta}$cya $_{83}$ cysG$^-_{73\cdot5}$

(4506-$\Delta$1 to 7)

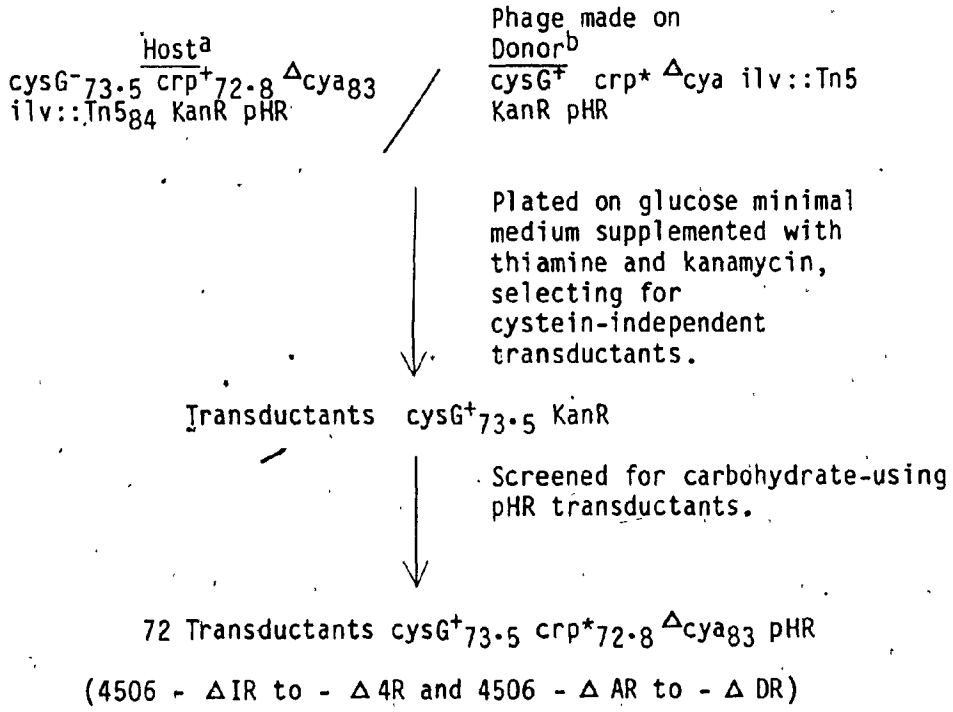
Further tested on minimal medium with lactose, or maltose or arabinose, with or without cAMP.

[a]Host strain used is 4506

[b]Donor strain used is JB-1

Note: The numbers after the gene symbols indicate map locations.

Figure 17. Scheme for transduction of crp* locus into cysG$^-$ $^{\Delta}$cya strain using cysG$^+$ as a selected marker.




[a]Host strain used is 4506-Δ1

[b]Donor strain used is NABR-1 to 4 and NABR-A to D.

Note: The numbers after the gene symbols indicate map locations.

each of these eight transductions, a certain percentage of the $cysG^+$ transductants was found to have a $cya^+$ phenotype, i.e., were pH-resistant and carbohydrate-utilizing (4506-ΔR1 to -ΔR4 and 4506-ΔAR to -ΔDR) (Table 16).

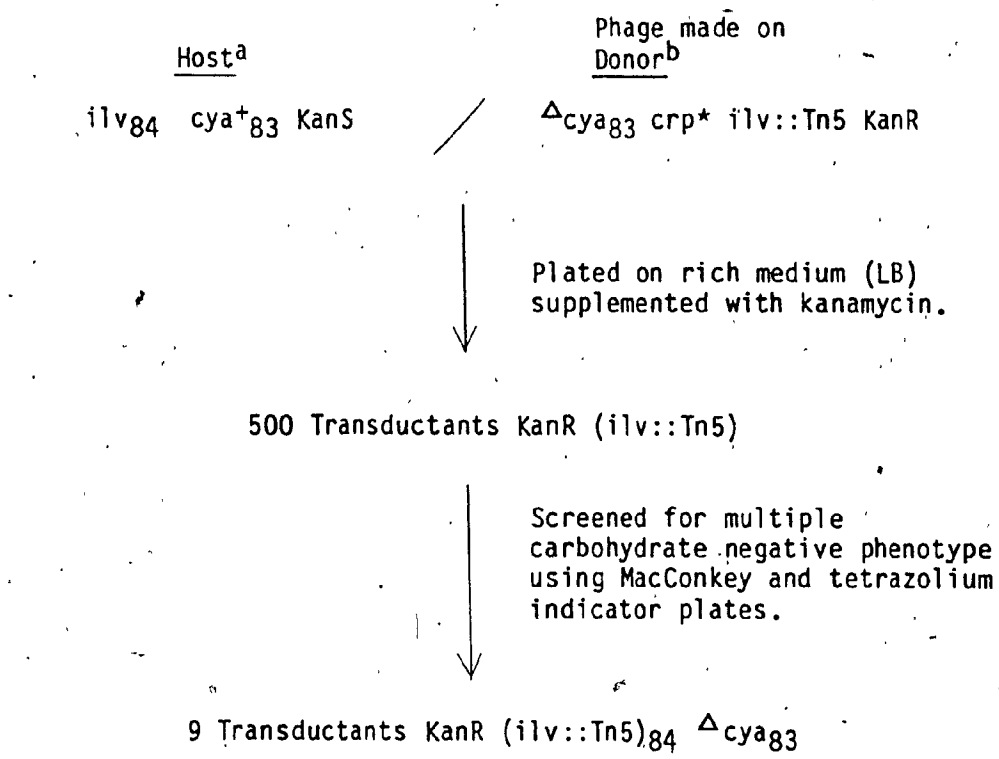
- Testing for $^{\Delta}cya$ mutation in 4506-ΔR1 strain:

To be certain that strain 4506-ΔR1 carries the *cya* deletion, although its phenotype is sugar-fermenting, the $^{\Delta}cya$ mutation was transferred from this strain into a $cya^+$ strain through transduction. The scheme of the experiment is illustrated in Figure 18. To perform this transduction, phage P1CM was grown on strain 4506-ΔR1. Strain NA-1 was used as recipient and kanamycin-resistant transductants were selected. Of 500 kanamycin-resistant transductants, nine were $^{\Delta}cya$, ie., carbohydrate non-utilizing and pH-sensitive. This result proves that strain 4506-ΔR1 does carry a $^{\Delta}cya$ mutation. Its cya+ phenotype then must be due to a mutation near *cysG*, ie., probably in *crp*.

C. Further genetic evidence for the involvement of the crp gene in cAMP/CRP mediated resistance to external alkaline pH:

If the CRP/cAMP complex is in fact involved in developing resistance to high pH, one would not expect to get any pH-resistant strains from a strain that carries a deleted *cya* gene as well as a deleted *crp* gene. To see if this is the case, two strains, CA8445 and CA8496, that carry double deletions and were as pH-sensitive (in liquid medium) as was strain NAB-1, were plated on glucose minimal

Figure 18. Scheme for transduction of $^{\Delta}$cya mutation from strain 4506-Δ1R to strain NA-1.




[a]Host strain used is NA-1

[b]Donor strain used is 4506-Δ1R

Note: The numbers after the gene symbols indicate map locations.

Table 16

Transduction frequency of the loci responsible for pH-resistance (crp) and cysG

| Host Strain | Donor Strain | Relevant Genotype: Host | Relevant Genotype: Donor | Selected Marker[a] | Unselected Marker[b] | Percentage of Strains Carrying Unselected Donor Marker |
|---|---|---|---|---|---|---|
| 4506-Δ1 | NABR-1 | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 29 |
| 4506-Δ1 | NABR-2 | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 27 |
| 4506-Δ1 | NABR-3 | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 14 |
| 4506-Δ1 | NABR-4 | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 24 |
| 4506-Δ1 | NABR-A | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 28 |
| 4506-Δ1 | NABR-B | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 20 |
| 4506-Δ1 | NABR-C | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 16 |
| 4506-Δ1 | NABR-D | cysG⁻ Δcya KanR ilv::Tn5 | cysG⁺ Δcya KanR ilv::Tn5 | cysG⁺ | crp* | 5 |

Note: [a] cysG⁺ scored as ability to grow on minimal medium without cystein.
[b] crp* scored on MacConkey plates (see text).

medium at pH 7.8. In three separate experiments, incubation up to 7 days did not give any pHR colonies.

Therefore, it was concluded from these experiments that the cAMP/CRP complex is most probably involved in pH-resistance, and that growth at high pH involves transcription at a cAMP-CRP-dependent promotor.

It was also possible to get a promotor mutation in the gene(s) that is responsible for the pH-resistance, such that it does not require cAMP/CRP for its expression (as has been found in the case of lac operon (37). But such mutants were not found when selection was made for obtaining pH-resistance mutants, either from the double deletion mutants or from mutants carrying single deletion mutation at the cya or crp loci.

## XII. Comparison Of ATPase Activity In The Mutant And Parent Strains:

It has been suggested, on the basis of the reduced growth rate of *cya* mutants, that these mutants may have a defective system for conservation of energy from metabolizable substrates (40). This thought was further strengthened by the low yield and pH-sensitivity of the $\Delta$*cya* mutant. Under aerobic conditions energy conservation in *E. coli* involves the generation of proton and ion gradients across the cytoplasmic membrane and utilization of these gradients to form ATP by the membrane bound ATPase. Problems in energy conservation could be due to problems in establishing or using the gradient(s) or both. Problems in using the gradient may be due to a deficiency in the ATPase activity. It has been previously reported that *cya* mutants have normal ATPase activity (40). To verify this for the mutant studied here, an assay was performed to measure the membrane bound ATPase hydrolytic activity in the mutant and parent strains grown on glucose minimal medium at pH 6.0 and 7.8. The results showed no significant difference between the two strains and the activity did not appear to be affected by pH in either of the two strains.

The ATPase activities in the mutant and the parent strains were 1.06 and 0.97 umoles $P_i$ released per minute per mg of membrane protein extracted from cultures grown at pH 6.0 (Table 17). In membrane preparations obtained from the mutant and the parent strains grown at pH 7.8 the ATPase activities were respectively 1.06 and 0.92 umoles $P_i$ released per minute per mg of membrane protein.

Table 17

Membrane bound ATPase activity in the mutant (NAB-I) and the parent (NA-1) strains

| Strain | Relevant Genotype | pH of the growth medium | ATPase activity (μmoles Pi per min per mg protein) Exp. I | Exp. II | mean ± S.D. |
|---|---|---|---|---|---|
| NA-1 | $cya^+$ | 6.0 | 1.00 | 1.12 | 1.06 ± 0.06 |
| | | 7.8 | 0.90 | 0.93 | 0.92 ± 0.02 |
| NAB-1 | $\Delta cya$ | 6.0 | 1.00 | 0.93 | 0.97 ± 0.04 |
| | | 7.8 | 0.90 | 1.03 | 0.97 ± 0.07 |

Note: Membrane preparations were made from cells grown in glucose minimal medium.
The pH of the growth medium was adjusted with HCl or KOH.
S.D. is standard deviation.

These results show that the $\Delta$ *cya* mutant has a normal ATPase activity and the reason for the reduced growth rates and yield is due to something other than the ATPase activity.

## XIII. Effect Of Environmental pH On The Rate Of Oxygen Uptake By Cells And Membrane Vesicles From The Normal And The Mutant Strains:

The adenyl cyclase mutants (cya$^-$) have been reported to have a reduced ability to use oxygen (40). Membrane preparations from cya$^-$ strains have shown a slower uptake of oxygen than those from a normal strain when NADH, succinate and D-lactate were given as substrates (40).

If the oxygen uptake rate of strain NAB-1 were both low and affected by the pH of the growth medium, this may explain the growth rate and yield data described in this work. That this is not so is shown by the following data on oxygen uptake by strain NA-1 (cya$^+$) and NAB-1 ($^{\Delta}$cya) grown at different pHs.

The results obtained show that the rate of oxygen utilization was not affected by the pH of the medium (Table 18) in either strain and was lower but not affected by the pH in the mutant.

The mutant and the parent strains grown and assayed at pH 6.0 used oxygen at a rate of 2.3 and 8.9 n moles/gm dry weight per hour, ie., the parent used oxygen about four times faster than the mutant. The rate of oxygen utilization by cells grown and assayed at pH 7.8 was very similar to those grown and assayed at pH 6.0, and was 2.2 and 9.4 units for the mutant and parent strains respectively. Cells grown at pH 6.0 and assayed at pH 7.8 or vice-versa did not show any significant difference in $O_2$ uptake from the cells grown and assayed at the same pHs (Table 18).

Table 18

Oxygen utilization by mutant and parent cells and vesicles at various pHs

| Strain | System used | pH of the growth medium | pH of the assay | Oxygen utilization (nmoles of $O_2$ utilized/gm dry wt/ hr) Exp. #1 | Exp. #2 | Exp. #3 | Exp. #4 | Average ± S.D. |
|---|---|---|---|---|---|---|---|---|
| NA-1 ($cya^+$) | Cells | 6.0 | 6.0 | 9.8 | 9.0 | 8.3 | 8.5 | 8.9 ± 0.7 |
| | | | 7.8 | 8.5 | 9.0 | 9.2 | nd | 8.9 ± 0.4 |
| | | 7.8 | 7.8 | 8.7 | 10.0 | 9.0 | 9.8 | 9.4 ± 0.6 |
| | | | 6.0 | 7.9 | 10.0 | 9.8 | nd | 9.2 ± 1.0 |
| NAB-1 ($\Delta cya$) | Cells | 6.0 | 6.0 | 2.6 | 2.4 | 2.1 | 2.1 | 2.3 ± 0.2 |
| | | | 7.8 | 2.1 | 2.1 | nd | nd | 2.1 ± 0.0 |
| | | 7.8 | 7.8 | 2.6 | 2.0 | 2.1 | 2.0 | 2.2 ± 0.3 |
| | | | 6.0 | 2.1 | 2.1 | nd | nd | 2.1 ± 0.0 |
| NA-1 ($cya^+$) | Vesicles | 6.0 | 6.0 | 2.1 | | | | |
| | | | 7.8 | 2.3 | | | | |
| NAB-1 ($\Delta cya$) | Vesicles | 6.0 | 6.0 | 0.9 | | | | |
| | | | 7.8 | 0.9 | | | | |

Note: Each result is an average of two assays.
nd not done.

Similarly, $O_2$ uptake by membrane vesicles (made from cells grown on glucose minimal medium at pH 6.0) remained unaffected by the pH, and was 2.1 and 2.3 units for the normal strain at pH 6.0 and 7.8 respectively, and 0.9 and 0.9 units for the mutant strain at pH 6.0 and 7.8.

Therefore, the $\Delta$ cya mutant uses oxygen more slowly than the parent strain, but the slow uptake of oxygen does not seem to be involved in the pH-sensitivity of the mutant strain.

## XIV. Determination Of Uptake Of $^{14}C$-Proline In Membrane Vesicles Prepared From The Mutant And The Parent Strains:

Experiments described in the previous sections show that the mutant strain NAB-1 had a normal ATPase activity and a reduced rate of oxygen uptake. Reduced oxygen uptake has also been seen in other cya mutants (40). The fact that the mutant strain oxidizes the substrate slowly may indicate that it is doing more fermentation than oxidation, as compared to the parent strain, and thus giving a lower cell yield. This will be reflected in an analysis of the end products from the two strains. However, this is not likely to account for slow growth at high pH, since the decrease in oxygen uptake rate is not greater at high pH. This suggests that other factors are involved. The mutant may have more difficulty in generating and/or maintaining a normal PMF at some pHs than at others. To investigate this, an indirect approach was made by comparing a function of PMF, the active transport of proline, at various external pHs by membrane vesicles made from the mutant and the parent strains. The active transport of proline is known to depend on PMF either directly (through symport with proton) (90, 173, 214) or, according to a recent report, indirectly (through symport with $Na^+$) (192). In whole cells, proline, after being transported, is metabolized further and thus, it becomes difficult to differentiate which one of the two steps, transport or further metabolism, is rate limiting. Vesicles, since devoid of cytoplasm, were chosen for the study of the transport.

It is well established that the electrochemical proton gradient ( $\Delta \mu H^+$ or PMF) is kept fairly constant over a wide range of external pHs, and a decrease in proton gradient ($\Delta$pH) as a function of external pH is compensated by a parallel increase in the electrical gradient (94, 147). The transport of some neutral substrates (for example, proline and lactose) depends on the PMF (173, 218) and therefore does not normally change significantly with the change in external pHs. The initial rates of the uptake of proline were measured to compare the magnitude of PMF, since a correlation seems to exist between the magnitude of PMF and the initial rate of transport of amino acids (173, 201, 218). Therefore, if in the mutant strain the PMF was significantly decreased at high pH, this might be reflected in a decreased initial rate of proline transport. The results presented in Table 19 show that indeed the rate of $^{14}C$-proline uptake of the normal strain was not affected at the external pHs tested, being 0.17, 0.16 and 0.17 n moles transported per minute per mg of protein at pH 5.5, 6.6 and 7.8, respectively. In the mutant, however, the uptake was lower than in the normal strain at all pHs, and this deficiency was greater at higher external pHs. At an external pH of 5.5, the transport was reduced to 59% of the rate seen in the normal strain. At pH 6.6, it was 50% and at pH 7.8, it was 35%. That is, the uptake in the mutant was low at pH 5.5, and decreased even further at pH 7.8 to 35% of its value at pH 5.5.

There is considerable variability in the results of transport

Table 19

Uptake of $^{14}C$-proline by ASC-PMS energized vesicles from the mutant and parent strains

| Strain | External pH ($pH_o$) | Initial rate (n moles taken in/min/mg protein) | |
|---|---|---|---|
| NA-1 ($cya^+$) | 5.5 | 0.17 | |
| | 6.6 | 0.16 | |
| | 7.8 | 0.17 | |
| | | | (% of uptake in the parent strain) |
| NAB-1 ($\Delta cya$) | 5.5 | 0.11 | 59% |
| | 6.6 | 0.08 | 50% |
| | 7.8 | 0.06 | 35% |

Note: Vesicles were made from cells grown on glucose minimal medium at pH 6. The experiment was done with eight preparations of vesicles.

assays with membrane vesicles. This is true for different samples of one vesicle preparation assayed on different days, as well as for assays performed at different times after the thawing of any one sample. Typical data for a vesicle preparation made on July 26th 1982, are given in Figure 19. One sample was thawed on August 24th, and assayed twice each at pH 5.5 and 7.8. The experiment was repeated with another sample on October 5th. Although there is considerable variation from assay to assay and from day to day, it is clear that the results at pH 7.8 are different from those at pH 5.5 (Fig. 19).

To minimize the effect of the random variation, each experiment was performed several times, and each time all determinations were done on one day at all three external pHs with vesicles from both parent and mutant strains. The results were fairly reproducible, in the sense that for each experiment, the transport by the vesicles from the mutant was low and sensitive to high external pH as compared to the pH insensitive transport exhibited by the vesicles from the normal strain. An example of one such set of assays is represented in Figure 20 and 21.

Since the rate of uptake varied among different preparations of vesicles, the experiment was repeated on eight individual preparations of vesicles and an average value was taken. Of eight individual preparations on each of the two strains, one preparation (preparation #2 from the normal cells) gave an extremely low rate of transport (Table 20, 21) while all other values were quite close to each other. Therefore, that particular set of observations was excluded from the

Figure 19: Uptake of proline by one preparation of membrane vesicles assayed on two different days.

Symbols: (A) assay pH 5.5

(B) assay pH 7.8

(●) assay done on 24th August 1982

(▼) assay done on 5th October 1982

Note: The strain used was NAB-1 ($\Delta$*cya*).
The vesicles were made from cells grown on glucose minimal medium at pH 6.0.
Vesicles prepared on 6th July, 1982.
Each assay contained 115 gm of protein.
The assay temperature was 25°C.

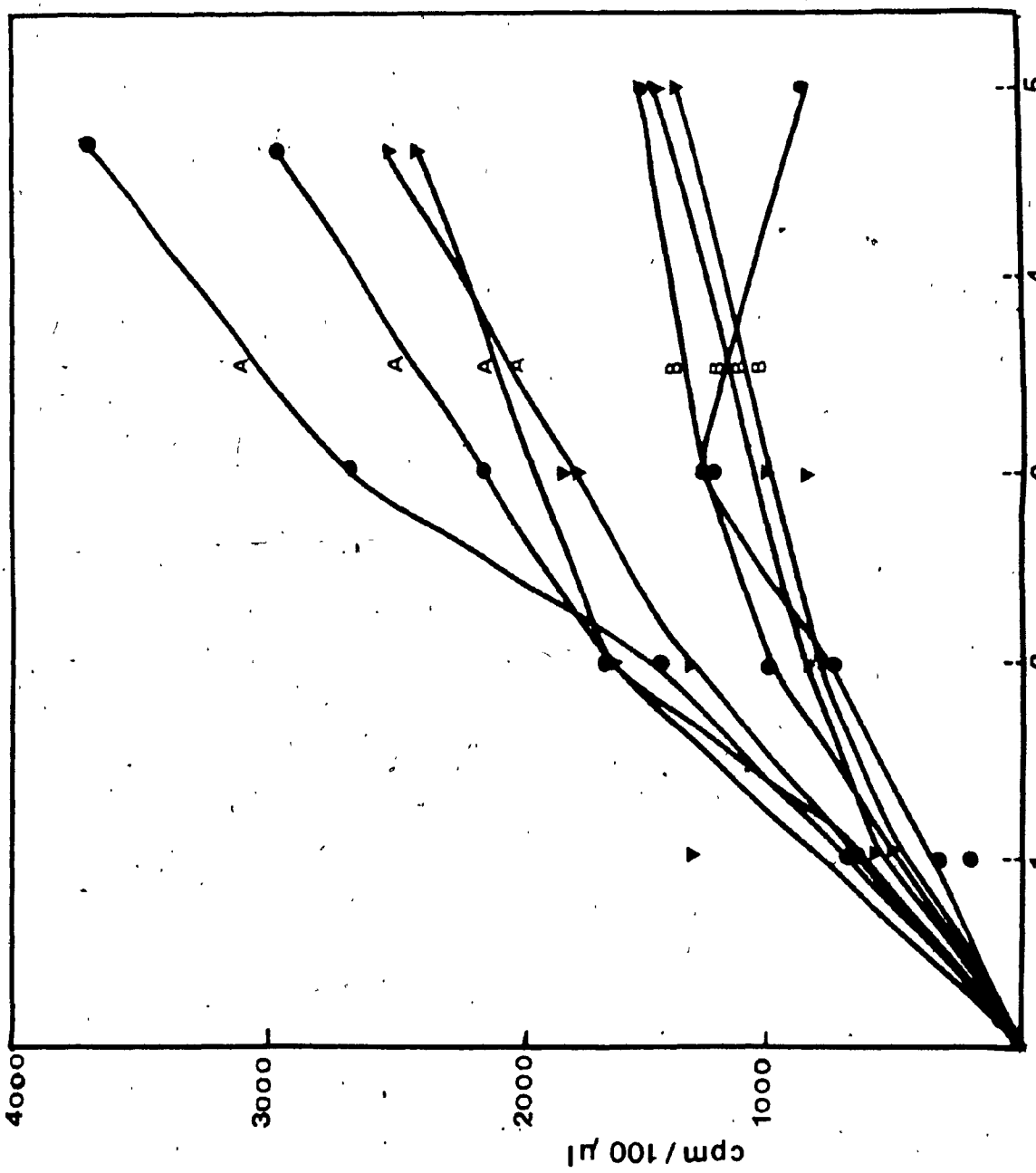



FIGURE 19

Figure 20: The effect of external pH on uptake of proline by membrane vesicles made from strain NA-1 ($cya^+$).

Symbols: (A) assay pH 5.5

(B) assay pH 6.6

(C) assay pH 7.8

Note: Vesicles were made from cells grown on glucose minimal medium at pH 6.0.
Each assay contained 0.105 mg of protein.
Assay temperature was 25°C.

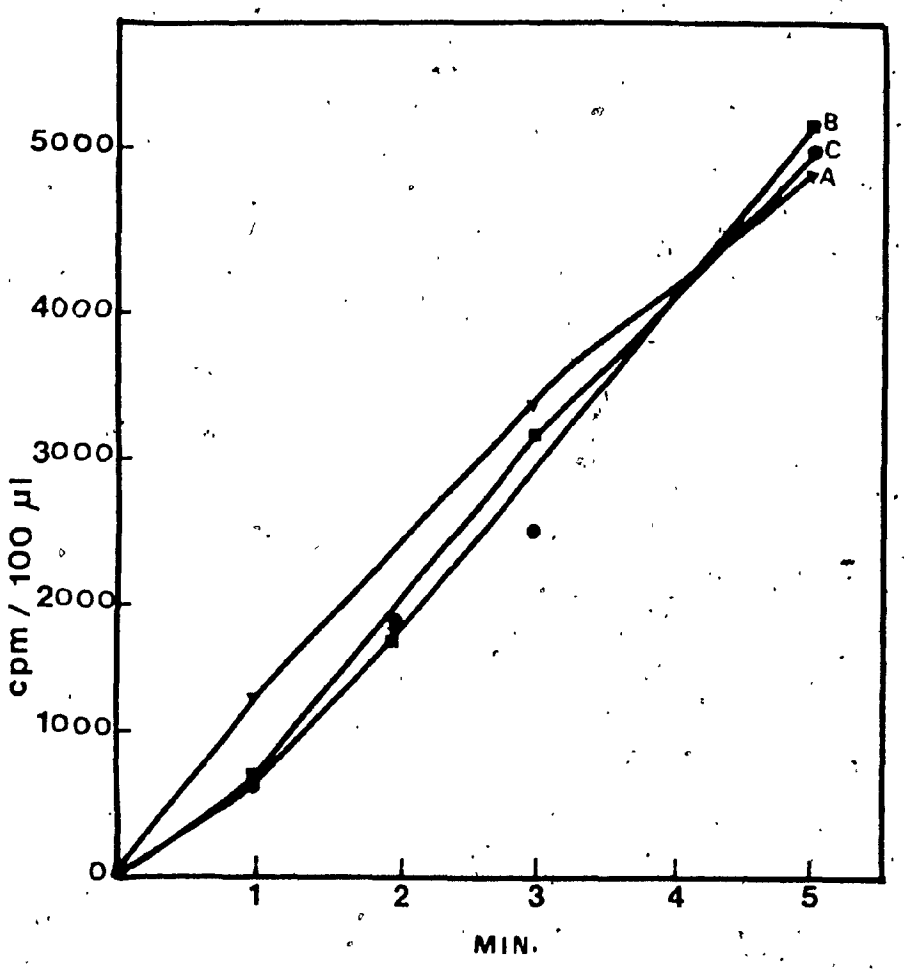



FIGURE 20

Figure 21: The effect of external pH on uptake of proline by membrane vesicles made from strain NAB-1 ($\Delta$cya).

Symbols: (A) assay pH 5.5

(B) assay pH 6.6

(C) assay pH 7.8

Note: Vesicles were made from cells grown on glucose minimal medium at pH 6.0.
Each assay contained 0.165 mg of protein.
Assay temperature was 25°C.

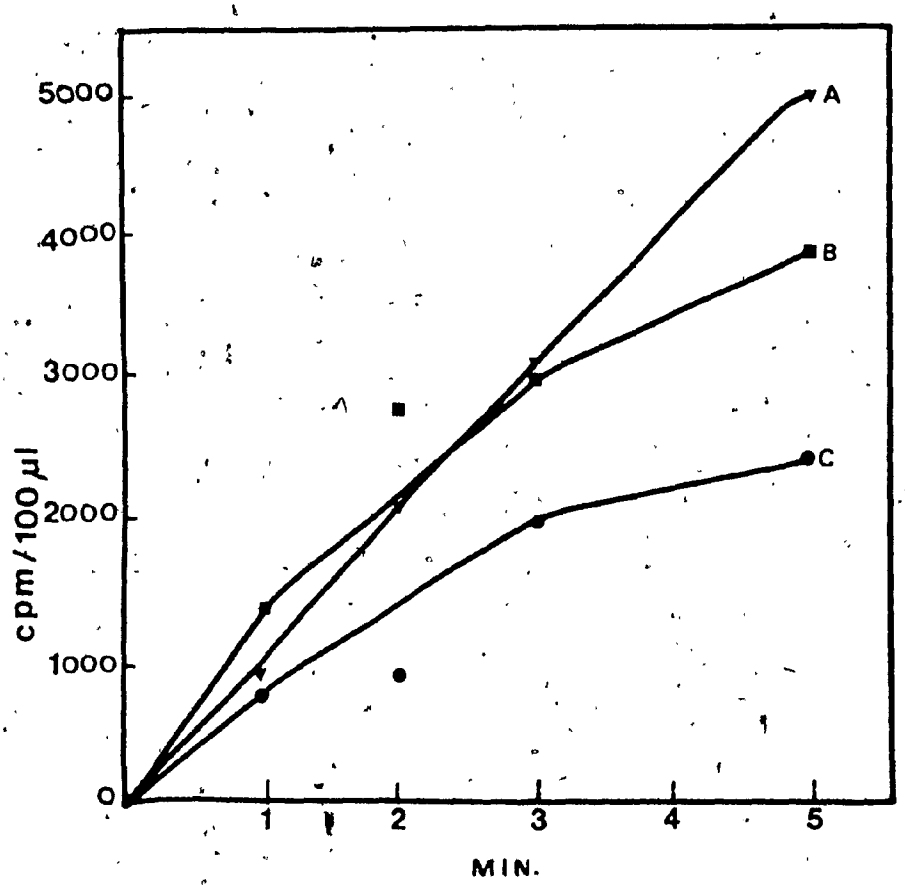



FIGURE 21

Table 20

Uptake of $^{14}C$- proline in an ASC-PMS energized system by eight individual preparations of vesicles

| Strain | Assay pH | Vesicle Prep. # | Initial Rate of $^{14}C$-proline uptake (cpm/min/mg protein) 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Average ± S.D. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NA-1 | 5.5 | | 8400 | 2090 | 9160 | 7700 | 8110 | 8750 | 8820 | 6960 | 8270 ± 750 |
| (cya$^+$) | 6.6 | | 8690 | 2060 | 9040 | 6770 | 8210 | 7000 | 10070 | 6740 | 8060 ± 1280 |
| | 7.8 | | 8320 | 1910 | 9610 | 7300 | 9000 | 6920 | 9090 | 8600 | 8410 ± 970 |
| NAB-1 | 5.5 | | 4920 | 5620 | 6460 | 5900 | 6520 | 6140 | 5070 | 3550 | 5520 ± 990 |
| ($\Delta$cya) | 6.6 | | 3680 | 4650 | 4460 | 3570 | 4280 | 5610 | 3370 | 3000 | 4080 ± 840 |
| | 7.8 | | 2770 | 2330 | 2440 | 3020 | 3230 | 4310 | 2400 | 1620 | 2760 ± 740 |

Note: Vesicles were made from cells grown on glucose minimal medium at pH 6.

Table 21

Uptake of $^{14}C$- proline in an ASC-PMS energized system by eight individual preparations of vesicles

| Strain | Assay pH | Vesicle Prep. # | Initial Rate of $^{14}C$-proline uptake (cpm/min) 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|---|
| NA-1 ($cya^+$) | 5.5 | | 800 | 280 | 960 | 1160 | 1010 | 720 | 790 | 560 |
| | 6.6 | | 830 | 280 | 950 | 1020 | 1030 | 560 | 920 | 540 |
| | 7.8 | | 790 | 260 | 1010 | 1100 | 1030 | 590 | 820 | 670 |
| Protein in the assay ( μg) | ---------- | | 95 | 135 | 105 | 150 | 125 | 85 | 90 | 80 |
| NAB-1 ($\Delta cya$) | 5.5 | | 570 | 930 | 540 | 620 | 750 | 640 | 450 | 320 |
| | 6.6 | | 420 | 770 | 370 | 380 | 490 | 580 | 310 | 270 |
| | 7.8 | | 320 | 390 | 200 | 320 | 370 | 450 | 220 | 140 |
| Protein in the assay ( μg) | ---------- | | 115 | 165 | 83 | 105 | 115 | 104 | 92 | 89 |

Note: Vesicles were made from cells grown on glucose minimal medium at pH 6.

calculation of the average rate of uptake. Including this would somewhat decrease the values for the parent strain, but would not alter the conclusions to be drawn.

From these observations it was concluded that the active transport of proline in the mutant is certainly lower than in the normal strain at all pHs tested, the difference being greater at high pH. Whether this pattern of transport indicates a general inadequate energization of the membrane at various pHs or is due to a change in the membrane structure and/or function that is specific to proline transport, was determined by assaying the transport of another amino acid, $^{14}$C-glutamate, using vesicles from the mutant and the parent strain. Transport of glutamate depends indirectly (through $Na^+$ gradient) on the PMF as it is symported with $Na^+$. The results obtained (Table 22) show that transport of glutamate is also lower in the mutant than the parent strain and that it is sensitive to high external pH, as was the case of proline transport. The transport of glutamate by the vesicles from the normal strain was unaffected by the external pH, being 0.12, 0.12 and 0.13 n moles of glutamate taken in per minute per mg of protein at pH 5.5, 6.6 and 7.8, respectively. However, in the mutant the uptake was 0.09, 0.06 and 0.04 n moles per min per mg of protein at pH 5.5, 6.6 and 7.8, respectively, which is about 75, 50 and 33% of the transport values for the parent strain. The fact that proline and glutamate do not share a common transporter and that the transport figures for glutamate are very similar to those seen with proline, supports the conclusion that the $\Delta$ *cya* mutant is

Table 22

Uptake of labelled amino acids by vesicles and cells, in strains NA-1 and NAB-1

| Strain | External pH | Rate of $^{14}$C-proline uptake by vesicles (n moles taken in/ min/mg protein) | Rate of $^{14}$C-glutamate uptake by vesicles (n moles taken in/ min/mg protein) | Rate of $^{14}$C-proline uptake by cells (n moles taken in/ min/mg protein) |
|---|---|---|---|---|
| NA-1 ($cya^+$) | 5.5 | 0.17 | 0.12 | 1.10 |
| | 6.6 | 0.16 | 0.12 | 1.17 |
| | 7.8 | 0.17 | 0.13 | 1.17 |
| NAB-1 ($\Delta cya$) | 5.5 | 0.11 (59%) | 0.09 (75%) | 0.62 (56%) |
| | 6.6 | 0.08 (50%) | 0.06 (50%) | 0.52 (44%) |
| | 7.8 | 0.06 (35%) | 0.04 (33%) | 0.24 (21%) |

Note: Uptake of amino acids by the vesicles was measured in an ASC-PMS energized system. Uptake by the cells was measured in a glucose energized system.

Percentages given are the percent of uptake seen in the parent strain.

deficient in membrane energization at all pHs, but particularly at high ones.

During the process of vesicle preparation, the cell is subjected to a variety of physical, chemical and enzymatic stresses. The possibility that the transport differences are due to a difference in response of the mutant and the parent cells to the process of vesicle-making, was tested by assaying for proline transport by whole cells. The results, presented in Table 22, show that the whole cells exhibit the same phenotype as vesicles, that is: the uptake in the mutant cells is slower and pH sensitive. Therefore, it seems that the difference in transport ability of the vesicles from the mutant and parent strain is not due to an artifact of the process of vesicle preparation.

These observations suggest that the PMF in the mutant is much lower than the normal strain, and that it decreases more with the increase in external pH.

As the PMF or proton electrochemical gradient ($\Delta \mu H^+$) is composed of two forces, an electrical gradient ($\Delta\psi$) and a proton gradient ($\Delta$pH), a low PMF could be due to low $\Delta\psi$ or $\Delta$pH or both. The fact that the uptake in the mutant strain is decreased with the increase in external pH, suggests the possibility that the $\Delta\psi$ might be affected in the mutant. This is based on the fact that at high external pHs, a normal strain keeps its PMF almost constant by increasing the contribution of $\Delta\psi$ to compensate for the decrease in $\Delta$ pH. Table 23 shows the approximate values of PMF, $\Delta\psi$ and $\Delta$ pH at various external

Table 23

Approximate values of PMF, $\Delta\psi$ and $\Delta$pH at various external pHs, in Escherichia coli

| External pH | PMF (mV) | $\Delta\psi$(mV) | $\Delta$pH (mV) |
|---|---|---|---|
| 5.5 | 200 | 95 | 105 |
| 6.6 | 190 | 130 | 60 |
| 7.8 | 150 | 150 | 0 |

Note: Values are calculated from Figure 1 of the review article by Padan et al. (147).

pHs in E. coli, calculated from Figure #1 in the review article by Padan et al. (156). Thus, in a normal strain, $\Delta\psi$ composes 45%, 70% and 100% of $\Delta\mu H^+$ at external pHs of 5.5, 6.6 and 7.8 respectively (while $\Delta$ pH composes 55%, 30% and 0% at external pHs of 5.5, 6.6 and 7.8). A strain having difficulty in generating and/or maintaining a $\Delta\psi$ will be expected to have a low PMF and the deficiency may become more noticeable at high external pHs, as proposed for the $^{\Delta}$cya mutant.

Further support in favor of the possibility that the $\Delta\psi$ value is affected in the mutant came from the study of proline transport in the presence of two antibiotics, valinomycin and nigericin, that, under certain conditions dissipate the $\Delta\psi$ and the $\Delta$ pH, respectively.

Uptake of proline in the presence of valinomycin (an ionophore that dissipates the electrical gradient in presence of sufficient $K^+$ ions in the medium) was measured in the presence of 50 mM $K^+$ in the assay at three external pHs in both strains, NA-1 and NAB-1. The results, presented in Table 24 show that at the concentration used, valinomycin decreased proline uptake in the wild type strain by 26-36% at all pHs tested. In the mutant, the decrease was 78-96% within the pH range from 5.5 to 7.8. Does this mean that the mutant is much more sensitive to valinomycin than the wild type? If one expects that a given concentration of valinomycin dissipates a certain percentage of the $\Delta\psi$, one would have to conclude that the mutant is more sensitive.

However, this is not a likely expectation. Valinomycin acts by inserting itself into the membrane as mobile $K^+$-carrier and dissipates

Table 24

Uptake of $^{14}C$-proline in the presence and absence of antibiotics: valinomycin and nigericin

| Strain | External pH | Proline uptake without antibiotics (n moles/min/mg protein) | Uptake in the presence of antiobiotics (n moles/min/mg protein) | | | |
|---|---|---|---|---|---|---|
| | | | Valinomycin | | Nigericin | |
| | | | | (% Inhibition) | | (% without) |
| NA-1 (cya$^+$) | 5.5 | 0.17 | 0.12 | (29%) | 0.13 | (77%) |
| | 6.6 | 0.16 | 0.11 | (35%) | 0.22 | (138%) |
| | 7.8 | 0.17 | 0.10 | (41%) | 0.24 | (141%) |
| | | | | (% Inhibition as compared to parent strain) | | (% without) |
| NAB-1 ($\Delta$cya) | 5.5 | 0.10 (59%*) | 0.04 | (76%) | 0.04 | ( 40%) |
| | 6.6 | 0.08 (50%*) | 0.02 | (87%) | 0.07 | ( 87%) |
| | 7.8 | 0.06 (35%*) | 0.01 | (94%) | 0.08 | (133%) |

* % of uptake seen in the parent strain.

a certain amount of the electrical gradient. The rate of $K^+$ leakage through valinomycin should be directly proportional to the absolute amount of valinomycin (within limits) and not to the absolute value of $\Delta\psi$. Therefore, it could be concluded that the mutant and the parent strains are equally sensitive to valinomycin and the apparent greater sensitivity of the mutant is probably due to a deficiency in $\Delta\psi$, a suggestion made earlier on the basis of the proline transport.

The uptake of proline was also measured in the presence of nigericin, a protonophore that inhibits the $\Delta$pH. The results, summarized in Table 24, provide further indirect evidence that the $\Delta\psi$ is lower in the mutant than in the normal strain. At the concentration used, nigericin decreased the uptake in the wild type strain to 77% at pH 5.5. In the mutant at pH 5.5, the uptake decreased to 40% (Table 24). This could mean that the mutant is more sensitive to nigericin than the parent strain. However, nigericin acts by inserting itself into the membrane as a mobile electroneutral carrier of $H^+$ and dissipates the $\Delta$pH. At pH 5.5, in the normal strain, total transport is 0.17 n moles. The amount of nigericin used dissipated approximately 23% of total transport in the wild type, so the transport was decreased by 0.04 n moles. If the mutant is equally sensitive to nigericin, it will lose 0.04 n moles of proline transported. The observed transport is only 0.04 n moles which is much less than the transport expected. Thus $\Delta\psi$ seems to be deficient in the mutant at pH 5.5.

At pH 6.6, the amount of nigericin used had no inhibitory effect

on transport in the wild type; in fact, the transport increased to 138%. In the mutant, the inhibition was much less than what was observed at pH 5.5, the transport being 87% of the transport seen in the absence of nigericin. At pH 7.8, in both strains, the transport increased to 141% for the wild type and 133% for the mutant. Thus, the mutant appears to behave normally in the presence of nigericin. The total uptake at pH 7.8 in the presence of nigericin was 0.24 n moles in the normal strain and 0.08 n moles in the mutant which is about 33% of the transport seen in the normal strain. Thus, the mutant appears to have approximately 33% of the transport activity seen in the wild type, as was observed in the proline uptake experiment described earlier.

These results provide indirect evidence that the pH sensitivity of the $\Delta$*cya* mutant is due to a deficiency of $\Delta\psi$ .

## XV. Determination Of Electrochemical Proton Gradient ( $\Delta \mu H^+$) In Vesicles And Cells From The $^{\Delta}$cya Mutant And Its Parent Strain:

Experiments described in the previous section suggested that the $^{\Delta}$cya mutant has difficulty in developing and/or maintaining a normal proton motive force (PMF or $\Delta \mu H^+$), and more so at high environmental pH. As indicated indirectly, the $\Delta\psi$ appears to be of lower magnitude in the mutant than in the normal strain. To investigate it further, attempts were made to measure the electrochemical proton gradient ( $\Delta \mu H^+$ or PMF) of the vesicles and cells from the mutant and the parent strain. This was approached by measuring each of the two components of $\Delta \mu H^+$, $\Delta\psi$ and $\Delta$pH. The electrical gradient ( $\Delta\psi$ ) in the case of the mutant was lower than the wild type strain at both pHs tested (Table 25, 26). The pH gradient ($\Delta$pH) was similar in the two strains (Table 25, 26).

Tritiated tetraphenyl phosphonium ion ($^3$H-TPP$^+$) was used as a probe to measure $\Delta\psi$. The data obtained for TPP$^+$ distribution as cpm per sample, from vesicles, is presented in Table 27. When TPP$^+$ was used at a concentration of 0.5 $\mu$M, the results were somewhat more reproducible at pH 7.8 than those obtained for pH 5.5. The pellet associated cpm were 269 $\pm$ 71 S.D. at pH 5.5 and 750 $\pm$ 48 S.D. at pH 7.8 for the normal strain and 35 $\pm$ 22 S.D. and 268 $\pm$ 121 S.D. for the mutant at pH 5.5 and 7.8, respectively. The mutant strain has much more variation in the cpm, especially at pH 5.5 when the $\Delta\psi$ was very low. These figures clearly show that the reliability of the

Table 25.

$\Delta\psi$, $\Delta$pH and PMF as a function of external pH in vesicles from strain NA-1 and NAB-1

| Strain | External pH (pHo) | $\Delta\psi$ (mV) | $\Delta$pH (mV) | PMF (mV) |
|---|---|---|---|---|
| NA-1 ($cya^+$) | 5.5 | 127 ± 7 | 124 ± 12 | 251 |
| | 7.8 | 146 ± 4 | 35 | 181 |
| NAB-1 ($^{\Delta}cya$) | 5.5 | 49 ± 21 | 122 ± 13 | 171 |
| | 7.8 | 100 ± 12 | 28 | 128 |

Note: Each determination is presented as ± standard deviation.

Table 26.

$\Delta\psi$, $\Delta$pH and PMF as a function of external pH in cells from strains NA-1 and NAB-1

| Strain | External pH (pHo) | $\Delta\psi$ (mV) | $\Delta$pH (mV) | PMF (mV) |
|---|---|---|---|---|
| NA-1 | 6.60 | 103 ± 15 | 58 ± 10 | 161 |
| (cya$^+$ | 8.21 | 188 ± 6 | +18 | 170 |
| NAB-1 | 6.60 | 60 ± 12 | 63 ± 7 | 123 |
| ($^\Delta$cya) | 8.25 | 170 ± 9 | +15 | 155 |

Note: Each determination is presented as ± standard deviation.

Table 27

Uptake of $^{3}H$-TPP$^{+}$ and the $\Delta\psi$ in vesicles at two $^{3}H$-TPP$^{+}$ concentrations in the assay

| Strain | External pH | $^{3}H$-TPP$^{+}$ in the assay ( $\mu M$) | Protein in the assay ( $\mu g$) | Pellet associated radioactivity per assay (cpm) | cpm out /$\mu l$ | $\Delta\psi$ (mV) |
|---|---|---|---|---|---|---|
| NA-1 (cya$^{+}$) | 5.5 | 0.5 | 95 | 269 ± 71 | 22 ± 3 | 107 ± 1 |
| | | 1.5 | 105 | 3140 ± 303 | 37 ± 2 | 148 ± 1 |
| | 7.8 | 0.5 | 95 | 750 ± 48 | 21 ± 3 | 135 ± 4 |
| | | 1.5 | 105 | 4239 ± 231 | 37 ± 1 | 157 ± 1 |
| NAB-1 ($^{\Delta}$cya) | 5.5 | 0.5 | 115 | 35 ± 22 | 21 ± 3 | 42 ± 23 |
| | | 1.5 | 165 | 140 ± 59 | 41 ± 1 | 57 ± 12 |
| | 7.8 | 0.5 | 115 | 268 ± 121 | 23 ± 5 | 93 ± 17 |
| | | 1.5 | 165 | 914 ± 19 | 40 ± 1 | 108 ± 1 |

Note: Each determination is presented as ± standard deviation.

technique decreased as the distribution ratio of $TPP^+$ across the membrane goes down because of the decrease in $\Delta\psi$.

In order to get a more conspicuous difference in the distribution of radioactivity, a higher concentration of $TPP^+$ (1.5 μM) was used. The results summarized in Table 27 show that for the vesicles from the wild type strain, at a higher concentration of $TPP^+$, the pellet associated radioactivity was less sensitive to the external pH, and was 3140 ± 303 S.D. and 4239 ± 231 S.D. at pH 5.5 and 7.8 for the vesicles from the normal strain. For the vesicles from the mutant strain at pH 7.8 (ie. at high $\Delta\psi$), the pellet associated cpm did not vary much at either concentration of $TPP^+$. Similarly, at pH 5.5, in the mutant strain, the pellet associated radioactivity at the two concentrations of $TPP^+$ did not vary much, and was very low in both cases (35 ± 22 S.D. at 0.5 μM $TPP^+$ and 140 ± 59 S.D. at 1.5 μM $TPP^+$ used). Whether such a small number can be used as an absolute measure of $\Delta\psi$ seems questionable. From these observations, it seems apparent that the validity of the technique varies greatly with different values of $\Delta\psi$ as well as with the concentration of $TPP^+$ used. In any case, the vesicles from the mutant strain always showed lower values for $\Delta\psi$ than the normal strain at both pHs tested. The absolute values of $\Delta\psi$ obtained, admitting the limitations of the method used, were 100 mV and 49 mV at pH 7.8 and 5.5, respectively, for the vesicles from the mutant, and 127 mV and 146 mV at pH 5.5 and 7.8 for the vesicles from the normal strain (Table 25).

The data obtained for the $TPP^+$ distribution as cpm per sample

from the cells, is presented in Table 28. At external pH 6.62 the $\Delta\psi$ in the mutant strain was much lower than the normal strain, being 60 $\pm$ 12 mV and 103 $\pm$ 15 mV, respectively. Similarly, at pH 8.2, the $\Delta\psi$ in the mutant strain was lower than that of the parent strain (being 170 $\pm$ 9 mV and 188 $\pm$ 6 mV, respectively) but the difference between the two was not as pronounced as it was at pH 6.6. In order to compare the $\Delta$pH component of the PMF in the mutant and parent strains, the pH gradient was measured directly. The results obtained clearly show that the magnitude of $\Delta$pH is the same at both pHs. In the vesicles at pH 5.5, the $\Delta$pH was 124 mV and 122 mV in the parent and mutant strains (Table 25). At pH 7.8, the $\Delta$pH was 28 mV and 35 mV for the mutant and the parent strains, respectively.

The distribution data in terms of cpm of $^{14}$C-benzoate ions are presented in Table 29. Using various concentrations of sodium benzoate (from 2-6 $\mu$M), the results were quite reproducible at pH 5.5. At pH 7.8 when the $\Delta$ pH is zero, a low concentration (2-4 $\mu$M) of benzoate gave almost no pellet associated counts (cpm being 37 $\pm$ 40 and 4 $\pm$ 203) and 6 $\mu$M gave higher cpm, 1229 $\pm$ 554 S.D. But all these numbers appear insignificant when compared with the pellet associated cpm at pH 5.5.

The data obtained for the sodium benzoate distribution in the cells are presented in Table 30. At external pH of 6.62, the $\Delta$ pH in the mutant and the parent strains were 58 $\pm$ 10 mV and 63 $\pm$ 7 mV, respectively. At pH 8.2 the $\Delta$pH in both strains were similar, being +18 mV and +15 mV respectively in the mutant and the parent strains.

Table 28

Uptake of $^{3}H$-$TPP^{+}$ and $\Delta\psi$ in cells from strains NA-1 and NAB-1

| Strain | External pH | $^{3}H$-$TPP^{+}$ cpm /0.8 ml cells | $\Delta\psi$ (mV) | |
|---|---|---|---|---|
| NA-1 ($cya^{+}$) | 6.59 | 2618 | 61.12 | |
| | | 5414 | 106.80 | |
| | | 4636 | 99.86 | |
| | | 5800 | 109.38 | |
| | 6.58 | 4713 | 107.24 | |
| | | 4120 | 102.81 | |
| | | 4447 | 105.35 | |
| | | 6220 | 115.91 | |
| | 6.62 | 6704 | 117.03 | |
| | | 1496 | -- | |
| | | 4220 | 99.69 | |
| | | 5515 | 111.15 | $\bar{X}$ = 103.2 ± 15.06 |
| NAB-1 ($\Delta$cya) | 6.61 | 2091 | 39.60 | |
| | | 2138 | 44.83 | |
| | | 1907 | -- | |
| | | 2379 | 61.59 | |
| | 6.58 | 2014 | 68.16 | |
| | | 2010 | 68.01 | |
| | | 2224 | 75.22 | |
| | | 2108 | 71.56 | |
| | 6.62 | 1938 | 62.24 | |
| | | 2057 | 66.61 | |
| | | 1619 | 44.52 | |
| | | 1843 | 58.11 | $\bar{X}$ = 60.04 ± 11.99 |

Table 28 (cont'd)

| Strain | External pH | $^{3}H$-TPP$^{+}$ cpm /0.8 ml cells | $\Delta\psi$ (mV) | |
|---|---|---|---|---|
| NA-1 (cya$^{+}$) | 8.20 | 35700 | 119.11 | |
| | | 20146 | 176.02 | |
| | | 35295 | 190.81 | |
| | | 36176 | 191.46 | |
| | 8.22 | 37991 | 183.05 | |
| | | 38051 | 183.09 | |
| | | 37310 | 182.55 | |
| | | 38119 | 183.14 | |
| | 8.22 | 40462 | 193.44 | |
| | | 41355 | 193.73 | |
| | | 40515 | 193.17 | |
| | | 42422 | 194.42 | $\bar{X}$ = 187.97 ± 6.05 |
| NAB-1 ($^{\Delta}$cya) | 8.30 | 26863 | 171.65 | |
| | | 26734 | 171.52 | |
| | | 14183 | 153.79 | |
| | | 28242 | 173.02 | |
| | 8.21 | 35684 | 181.84 | |
| | | 31209 | 178.92 | |
| | | 35501 | 181.69 | |
| | | 34707 | 181.07 | |
| | 8.23 | 22181 | 161.84 | |
| | | 21871 | 161.02 | |
| | | 23574 | 163.19 | |
| | | 24136 | 163.67 | $\bar{X}$ = 170.18 ± 9.44 |

Table 29

Uptake of $^{14}$C-benzoate ions and ΔpH in vesicles at two of $^{14}$C-benzoate concentration in the assay

| Strain | External pH | $^{14}$C-benzoate in the assay ( μM) | Protein in the assay ( μg) | Pellet associated radioactivity per assay (cpm) | cpm out /μl | ΔpH (mV) |
|---|---|---|---|---|---|---|
| NA-1 (cya$^+$) | 5.5 | 2 | 95 | 3211 ± 1240 | 115 ± 8 | 124 ± 12 |
| | | 6 | 105 | 10812 ± 317 | 324 ± 2 | 126 ± 1 |
| | 7.8 | 2 | 95 | 37 ± 40 | 112 ± 6 | 2 ± 4 |
| | | 6 | 105 | 1182 ± 106 | 389 ± 8 | 68 ± 4 |
| NAB-1 ($^{\Delta}$cya) | 5.5 | 4 | 115 | 5931 ± 860 | 126 ± 13 | 133 ± 5 |
| | | 6 | 165 | 12900 ± 613 | 341 ± 8 | 121 ± 1 |
| | 7.8 | 4 | 115 | 4 ± 203 | 137 ± 21 | 16 ± 6 |
| | | 6 | 165 | 1229 ± 554 | 359 ± 20 | 40 ± 21 |

Note: Each determination is presented as ± standard deviation.

Table 30

Uptake of $^{14}$C-benzoate ions and Δ pH in cells from strains NA-1 and NAB-1

| Strain | External pH | $^{14}$C-benzoate cpm /0.8 ml cells | Δ pH (mV) | |
|---|---|---|---|---|
| NA-1 (cya$^+$) | 6.65 | 2189 | 2.49 | |
| | | 6972 | 70.44 | |
| | | 3488 | 41.48 | |
| | | 5886 | 63.03 | |
| | 6.62 | 5950 | 59.70 | |
| | | 4652 | 57.41 | |
| | | 3441 | 45.22 | |
| | | 2150 | 18.10 | |
| | 6.63 | 3914 | 55.29 | |
| | | 2202 | 28.59 | |
| | | 5573 | 67.83 | |
| | | 4877 | 63.96 | $\bar{X}$ = 58.26 ± 9.7 |
| NAB-1 ($^{\Delta}$cya) | 6.65 | 3426 | 55.33 | |
| | | 4860 | 68.67 | |
| | | 4944 | 69.27 | |
| | | 4605 | 66.75 | |
| | 6.62 | 4542 | 60.68 | |
| | | 4835 | 62.64 | |
| | | 3982 | 56.46 | |
| | | 4115 | 57.53 | |
| | 6.63 | 7306 | 76.86 | |
| | | 3581 | 54.45 | |
| | | 4916 | 64.87 | |
| | | 4594 | 62.71 | $\bar{X}$ = 63.01 ± 6.69 |

Table 30 (cont'd)

| Strain | External pH | $^{14}$C-benzoate cpm /0.8 ml cells | ΔpH (mV) | |
|---|---|---|---|---|
| NA-1 (cya$^+$) | 8.21 | 1496 | | |
| | | 1771 | | |
| | | 1932 | | |
| | | 2352 | -26.38 | |
| | 8.21 | 2538 | | |
| | | 1818 | | |
| | | 2043 | | |
| | | 1659 | -30.12 | |
| | 8.19 | 2736 | | |
| | | 2064 | | |
| | | 1697 | | |
| | | 2015 | + 3.00 | $\bar{X}$ = -17.83 |
| NAB-1 ($\Delta$cya) | 8.13 | 2752 | | |
| | | 2774 | | |
| | | 2118 | | |
| | | 765 | -15.4 | |
| | 8.20 | 1482 | | |
| | | 2468 | | |
| | | 1872 | | |
| | | 1765 | -52.77 | |
| | 8.20 | 2342 | | |
| | | 1567 | | |
| | | 1979 | | |
| | | 2206 | +24.80 | $\bar{X}$ = -14.46 |

Therefore, these results provide direct evidence for the hypothesis made earlier that the electrical gradient in the $\Delta$*cya* mutant is lower than in the wild type at all pHs tested.

# DISCUSSION

The work described in this dissertation deals with a novel characteristic of a highly pleiotropic adenyl cyclase deletion mutation ( $^{\Delta}$cya) in Escherichia coli K-12: the sensitivity of the mutant to high environmental pH. This phenomenon of pH sensitivity is explained by proposing that the cya mutant is unable to generate and/or maintain a normal electrical gradient ( $\Delta\psi$ ) across the cytoplasmic membrane.

The line of thought on which this hypothesis was based is the following. The rate of growth of the cya mutant on several substrates (glucose, alanine, glucosamine, gluconate and pyruvate) at external pHs ranging from 6 to 7.8, decreased greatly as a function of an increase in pH. The yield of cell material per unit substrate also decreased dramatically with pH. The decrease in yield suggested that the substrate was being used less efficiently at higher pHs. This inefficiency in the utilization of the substrate by the mutant could be either because of the incomplete breakdown of the substrate or due to an alteration in the process of energy conservation. The first possibility that the incomplete breakdown of the substrate was the reason for the decreased yield can be verified by an analysis of the end products of metabolism. However, since the decrease in yield was observed with all the substrates tested, it seems that the mutant is deficient in some function that is needed for growth on all substrates. Moreover, since the external pH plays a very important

and direct role in aerobic energy metabolism, a more plausible explanation can be given based solely on a deficiency in the process of energy conservation. The work presented here was performed to explore this possibility.

According to recent theory, most energy conservation under aerobic conditions occurs through the development of a proton motive force (PMF) across the cytoplasmic membrane. The PMF is composed of two components, a proton gradient ($\Delta$pH) and an electrical gradient ($\Delta\psi$). A decrease in efficiency of energy transduction may then indicate a malfunction of the ATPase, or of the reactions generating and/or maintaining the electrical or proton gradient.

The cya mutant showed normal ATPase activity as judged by the ATP hydrolysing activity in the membrane preparations. Unlike the uncB mutants with a defect in the $F_0$ subunit of ATPase, cya mutants have been reported to be able to make ATP from an artificial electrochemical gradient (40). It therefore seemed more likely that the PMF was altered. That this is so, is indicated by the fact that proline transport, which is energized by the PMF, either directly through proton symport (173) or, according to a recent report, indirectly through the sodium gradient (192), is lower in the cells and membrane vesicles of the cya mutant than in the parent strain, this deficiency being much greater at high environmental pH.

The PMF remains roughly constant as a function of external pH. The contributions of its two components however vary greatly. At low external pH, the contribution of $\Delta$ pH to the PMF is about 55%. At pH

7.8, $\Delta$pH makes no contribution to the PMF, which consists entirely of the electrical gradient ( $\Delta\psi$ ). Thus, a deficiency in the development and/or maintenance of $\Delta\psi$ would be consistent with an increase in the difficulties in function as the external pH increases from 6 to 7.8, but a deficiency in the development and/or maintenance of $\Delta$pH would not.

The prediction that the $\Delta\psi$ might be affected was tested by estimating the effects of the antibiotics, valinomycin and nigericin, which interfere with the maintenance of $\Delta\psi$ and $\Delta$ pH respectively. The PMF was also measured directly. The results support the explanation proposed.

Part 1. A Summary Of The Proof That Strain NAB-1 Is A Typical $\Delta$*cya* Mutant.

The characterization of the physiology of the $\Delta$*cya* strain depends on choosing an appropriate strain. A $\Delta$*cya* mutant (JB-1) was therefore obtained from a reliable investigator, J. Beckwith, and transduced into a derivative of our regular laboratory strain of *Escherichia coli* K-12, strain NA-1. The donor strain as obtained from him also carried a transposon (Tn5) integrated into the *ilvA* gene, one minute away from the *cya* mutation.

Because this transposon specifies kanamycin resistance, cells from the NA-1/JB-1 transduction were selected on a rich medium supplemented with kanamycin. The recipient strain, which is kanamycin sensitive, was counterselected on this medium. Only transductants that carried the insertion element would be kanamycin resistant and thus appear on these plates. Since the Tn5 is inserted close to the *cya* mutation, some of the kanamycin-resistant transductants should also carry the *cya* mutation. These were located by testing on MacConkey plates with a combination of lactose, maltose and arabinose. Colonies which appeared white, and were thus unable to form acid, were also shown to be non-fermenters on tetrazolium lactose and tetrazolium arabinose plates. All the isolates which were taken to be *cya* mutants were able to use the carbohydrates tested if cyclic adenosine monophosphate (cAMP) was provided.

The sugar-fermenting characteristics of the strains isolated, as

well as their method of construction, suggest that they contain the same $\Delta$ *cya* mutation as the original Beckwith strain. The extent of linkage between *cya* and *ilvA* (approximately 3%) was considerably lower than expected. It seems likely that this is due to two factors, first that the donor strain carried the Tn5 insertion, approximately 5200 base pairs long (98, 99), in the *ilvA* gene and second, that the recipient strain has a deleted *ilvA* gene. Since the transducing phage must contain the Tn5 element in order to transfer kanamycin resistance, and since it can transfer only 2 minutes of DNA (10), it might be expected that the co-transduction would be reduced in a transduction performed in this manner. The under-representation of *cya* mutants could also be due to the fact that they grow much more slowly than the parent strain.

In so far as cAMP restores the ability of the mutant to use lactose, maltose and arabinose, it is clearly a *cya* mutant. To be certain that this is so, the position of the mutation in the strain selected for further study, strain NAB-1, was genetically characterized. When phage from a wild type strain were used to transduce strain NAB-1 (*ilv*::Tn5) to isoleucine independence, 57% of the transductants were $cya^+$. This is in agreement with the known distance between *ilvA* and *cya* (1 minute), (11, 26, 35, 217) and confirms that the mutation in strain NAB-1 is in fact a *cya* mutation.

Part. 2 Growth Of The $\Delta$cya Mutant Is Sensitive To pH.

Strain NAB-1 grew more slowly than its parent at every pH tested. At pH 6, the lowest pH tested, the doubling times (d.t.) were 135 and 63 minutes, respectively. The deficiency of the mutant increased with pH, so that at pH 7.8 it was barely able to grow (d.t. 270 minutes), while the parent was scarcely affected (d.t. 68 minutes).

That the slow growth was due to the deficiency of cAMP was proved by the restoration of the normal growth rate on addition of exogenous cAMP. Growth rate measurements at pH 6.4 in the presence of varying amounts of cAMP indicated that exogenous cAMP at 1 to 2.5 x $10^{-3}$ M was able to restore the growth rate to that of the wild type. At pH 7.8 the concentration of cAMP needed to restore a near normal growth was around 2.5 to 5.0 x $10^{-2}$ M. It seems that at alkaline external pH, the $\Delta$cya mutant requires more exogenous cAMP than the amount needed to restore the normal growth at acidic pH. The significance of this difference is not obvious. It may be that the cell needs more cAMP at basic external pHs than at acidic ones. However, it may not be so since the observation was made when cAMP was used exogenously and the mutants deficient in cAMP require a very high concentration of exogenous cAMP as compared to the concentration present in a normal cell. However, the slow growth rates, at both pHs, were restored to normal, showing that they were due to the deficiency of cAMP.

Further evidence that the sensitivity of growth of the cya mutant

to pH is due to the cya mutation itself, was provided by the study of pH resistant derivatives. The mutation restoring the ability of the cya mutant to grow at high pH proved to be located in the crp gene. This location was indicated by linkage to cysG (11).

Moreover, all these pH-resistant mutants were able to ferment both lactose and arabinose, indicating that several cAMP-dependent functions were restored simultaneously with pH-resistance. Strain NAB-1 carries a deletion in cya; this must also be true of the pH-resistant ($pH^R$) mutants. That this is so was proved by the transduction of $\Delta$cya mutation from one of these $pH^R$ strains, into a cya$^+$ strain. As mentioned in the introduction, a mutation in crp, altering the structure of the catabolite repressor protein, can allow that protein to function even in the absence of cAMP (20, 37, 197). This altered protein could then restore the activity of several promoters.

The fact that a crp mutation restores growth at high pH suggests that growth at high pH involves some function(s) that is dependent for transcription on the cAMP/CRP or CRP system. This involvement of crp was also indicated by the fact that a pH resistant strain could not be selected from a strain that carried deletions both in cya and crp. Although it might be possible to get a pH-resistant ($pH^R$) mutant from such a double deletion strain, in which the promotor for the gene(s) responsible for pH-resistance, is mutated in such a way that it does not need cAMP for the transcription, such a mutation was not obtained through the selection procedure used.

In summary then, these findings suggest that the cAMP/CRP complex is required for growth at a normal rate in glucose-minimal medium at every pH tested. Though it is needed at all pHs, as judged by the slow growth of the mutant at even pH 6, a deficiency in cAMP has a much more marked effect at higher pH.

The evidence presented suggests that the underlying cause of slow and pH-sensitive growth of the cya mutant is an alteration of energy metabolism. The slow growth (but not the pH-sensitivity) has been described by Dobrogosz and his collaborators (50). They classified growth on 20 substrates, concluding that a cya mutant grows slowly on sugars transported by the phosphotransferase system (PTS), and does not grow on non-PTS substrates, except pyruvate and gluconate, which it uses slowly (50). The slow growth was interpreted as a deficiency in the formation of the phosphotransferase system in cya mutants. However, the reason for slow growth on the two non-PTS substrates, pyruvate and gluconate, was not explained.

The mutant studied here grew slowly on the PTS-sugars, glucose and glucosamine, and on the non-PTS substrates, L-alanine, D-alanine, gluconate and pyruvate. It grew at an almost normal rate on glycerol, a compound which was not used by Dobrogosz's mutant (50). The cya mutant then is hindered in the use of a variety of carbon sources which do not share a common transport system. It seems then that the defect must lie not in the mechanism of transport per se, but in something common to the metabolism of all substrates.

Glycerol is the only compound which supports a near normal growth

rate of the cya mutant, and it does so at all pHs. It is also the only substrate, of those tested, which is transported by facilitated diffusion without a requirement for energy (110). All substrates which support slow and pH-sensitive growth, require energy for transport, either in the form of a proton motive force (PMF) or as phosphoenolpyruvate. The hypothesis presented here is that the cya mutant has difficulties in developing and/or maintaining a normal PMF and that this inefficiency decreases the rate of transport of these substrates, and consequently the growth rate of the mutant.

This hypothesis is supported by the fact that the yield of cell material from glycerol is lower and pH-sensitive in the mutant, even though the growth rate is not affected. The yield from glucose is, of course, also low and pH-sensitive. This can be understood as a deficiency in the energy metabolism, such that a given amount of substrate, once transported into the cell, provides less energy to the mutant cell than to the parent. Then glycerol would enter almost normally (normal growth rate) but would be used inefficiently (low yield). Other sugars would enter slowly (decreased rate) and also be used inefficiently (decreased yield). L-alanine, D-alanine and gluconate are all known to be transported by PMF-dependent systems (173). The PTS depends on phosphoenolpyruvate (which is derived by glycolysis) for its function, and it seems reasonable that the availability of phosphoenolpyruvate will depend on the availability of the energy source. Various investigators have suggested that transport carriers change their activity in response to the

energization of the membrane (212, 213). This was reviewed by Robillard and Konings (177), who proposed that the "activity of the PTS is regulated by the redox potential in the membrane, which changes in response to the PMF".

In summary, then, it is proposed that the reduction in growth rate and yield is due to a difficulty in energy transduction, and that this difficulty is accentuated at higher pHs.

## Part 3. Identification Of The Defect In Energy Transduction In The $\Delta$cya Mutant.

If the $\Delta$cya mutant has a defect in energy transduction, what can that defect be? According to the widely accepted formulation of energy conversion proposed by Mitchell (134), under aerobic conditions the proton motive force across the energy transducing membrane serves as the immediate source of energy that is used for biological functions: for making ATP through membrane-bound ATPase, for transport of ions and substrates against concentration gradients, and for motility. In these terms, the inefficient conversion of energy by the $\Delta$cya mutant is most likely due to a deficiency in the proton motive force, or to an alteration in the ATPase activity (the PMF being the interphase in the process of energy conversion, and ATP being the major energy currency of the organism).

It is known that a defect in ATPase will reduce the yield of cell material. Thus, an uncA mutant, having a defective catalytic unit of ATPase, and an uncB mutant, having a defective structural unit ($F_0$) of ATPase, yield less cell mass per unit of substrate than their parent (143). However, the ATPase activity in the $^{\Delta}$cya mutant studied here appeared to be normal. This is also true of the strain studied by Dill and Dobrogozs (40). These workers have also shown that the cya mutant cells are normal in their ability to use an artificially developed proton motive force, indicating that $F_0$ protein is not altered (40). Since ATPase is a very complex enzyme, and was studied here by assaying for the ATP hydrolysis in the membrane preparations, minor alterations in the ATPase of the cya mutant are still not excluded. However, there is no reason to suppose that they exist, and a complete explanation can be given in terms of a defect in the PMF.

Though the PMF remains roughly constant with pH, the relative contributions of its components, $\Delta\psi$ and $\Delta$pH, vary considerably with pH. A defect in any of these two components may therefore result in a pH-sensitive phenotype. The internal pH ($pH_i$) of an E. coli cell is maintained at about 7.8 (146, 147). When the cell grows at pH 7.8, it uses only one component of the PMF, the electrical gradient ($\Delta\psi$) and has no $\Delta$pH. At lower external pHs, the cell uses both components, the share comprised by the $\Delta$pH increasing as the external pH decreases. If the cell had trouble in maintaining a constant $pH_i$, and therefore a $\Delta$pH, its growth should be affected by the external pHs. At acidic external pHs it should be affected because of the excessive

acidification of the cytoplasm. Thus, its growth should be better at external pHs that are closer to 7.8. However, if it had trouble maintaining the electrical gradient, $\Delta\psi$, its growth would be slow, and pH-sensitive, with difficulties intensified at high pH, the case described for the $^{\Delta}$cya mutant.

A decrease in the electrical gradient ( $\Delta\psi$ ) then is consistent with the observed phenotype. That the PMF (and $\Delta\psi$) actually is reduced is suggested by the decrease in transport of two amino acids, a function energized by the PMF, in membrane vesicles of the $^{\Delta}$cya mutant. In the normal strain, proline transport was affected very little by external pH (0.17, 0.16 and 0.17 n moles transported per min per mg protein at pH 5.5, 6.6 and 7.8, respectively), (Table 22). In the mutant, however, transport is lower at all pHs, and is considerably affected by pH (being 59, 50 and 35% of the transport seen in the parent strain at pH 5.5, 6.6 and 7.8 respectively). The results are very similar for the transport of glutamate, an amino acid that is transported with $Na^+$ using the $Na^+$-gradient which in turn depends on the PMF. In the parent, the transport is essentially unaffected (0.12, 0.12 and 0.13 n moles/min/mg protein). In the mutant, the transport is lower and pH-sensitive (75, 50 and 33% of the parent at the three pHs).

Now, transport of these amino acids in membrane vesicles, directly or indirectly (through the $Na^+$ gradient), is energized by the PMF (173). They do not share a common transporter. The process of transport of these amino acids in vesicles depends upon an energy

source and a specific carrier. Since the specific carriers are not likely to be affected, it seems that the cya mutant must be deficient in the energization of the process of transport.

The whole-cell experiments also support the conclusion that transport in the cya mutant is deficient; the vesicle experiments locate the defect at a stage of energization.

The sensitivity to high pH indicated that it is the $\Delta\psi$ which was affected. The question of whether the $\Delta\psi$ in the mutant is really different has been approached by measuring the effect of ionophores on proline transport. The antibiotic valinomycin in the presence of high $K^+$ concentration kills its target cells by making them specifically leaky to cations, $K^+$ and $Rb^+$, thus dissipating the PMF (138, 201). It does this by inserting itself into the membrane as a mobile carrier, catalysing the electrical uniport of these cations (138). Now, setting up a $\Delta\psi$ depends on keeping the cations out of the cell. Valinomycin prevents the formation of a $\Delta\psi$ by providing free passage to $K^+$.

In the experiments presented here, the concentration of valinomycin used (5 μM) did not totally abolish the $\Delta\psi$. In fact, it decreased transport in the parent strain by only 41% at pH 7.8 where only $\Delta\psi$ is functioning.

The rate of leak at a given concentration of valinomycin should be the same in the mutant and the parent, since the number of $K^+$ uniporters created should be the same. If the mutant $\Delta\psi$ were the same as the parent $\Delta\psi$, then the percent inhibition by valinomycin

should also most probably be the same. But if the $\Delta\psi$ is lower, then the absolute value of the leak will not change, the extent of cation extrusion will be much lower, and the inhibition due to valinomycin will be much higher. This is indeed the case, since valinomycin inhibits the mutant 94% at pH 7.8.

The valinomycin experiment then suggests that the mutant maintains a lower $\Delta\psi$ than the parent. This suggestion is strengthened by the fact that nigericin has exactly the same effect in the parent and in the wild-type. Nigericin, also an antibiotic that inserts into the membrane as a mobile electroneutral carrier of proton, interferes with the maintenance of $\Delta$pH. The fact that the mutant and the parent respond the same way, indicates that they have similar $\Delta$pHs — as was suggested by the data considered earlier.

The $\Delta$pH and the $\Delta\psi$ were both measured directly in the cells and the membrane vesicles of the mutant and the parent strains. In the vesicles, the parent strain showed a PMF of 251 mV at pH 5.5 and 181 mV at pH 7.8, while the mutant showed 171 and 128 mV at pH 5.5 and 7.8, respectively. In the cells from the parent strain the PMF was 161 and 170 mV at pH 6.6 and 8.21, respectively. In the mutant cell it was 123 and 155 mV at pH 6.6 and 8.25, respectively. In so far the results of these measurements can be relied on the mutant showed a lower PMF at both pHs in the cells as well as in the vesicles.

The $\Delta$pH of the mutant and the parent strains were roughly similar at both pHs. In the vesicles from the mutant and the parent

strains, it was respectively 122 and 124 mV at pH 5.5 and, 28 and 35 mV at pH 7.8. In the cells it was 63 and 85 mV at pH 6.6 and, +17 and +14 mV at pH 8.2. However, the $\Delta\psi$ values for the two strains were very different. At pH 5.5 the $\Delta\psi$ in the vesicles from the parent strain was 127 mV. At the same pH the $\Delta\psi$ in the mutant strain was considerably lower (49 mV). Similarly, the parent and the mutant cells respectively had a $\Delta\psi$ of 103 and 60 mV at pH 6.6. At pH 7.8, vesicles from the parent showed a $\Delta\psi$ of 146 mV and the mutant 100 mV. In cells from the parent and the mutant strains at pH 6.6 it was 187 and 170 mV, respectively. This showed that the mutant can set up a $\Delta\psi$, but that it is of lower magnitude than that of the parent strain.

The fact that the mutant can establish some $\Delta\psi$ at pH 7.8 is in agreement with the earlier finding that the mutant can transport some proline and glutamate at pH 7.8 (35 and 33% of the parent value). The decrease in the $\Delta\psi$ of the mutant would probably account for the decrease in the transport; however the exact relationship between the $\Delta\psi$ values and the transport rates in E. coli is not yet known. Recently Trombé et al. (201) have isolated a mutant of Streptococcus pneumoniae that has an altered electrical gradient. This mutant, like the $^{\Delta}$cya mutant, could maintain a normal $\Delta$ pH, had normal ATPase properties and showed a reduction in the velocity of the $\Delta\psi$-energized uptake of some amino acids. This mutant also showed a pH-dependent sensitivity, of amino-acid transport, to valinomycin. However, the $\Delta\psi$, in this mutant, at different pHs was not measured to correlate

the ability of transport with the magnitude of $\Delta\psi$.

There is a great deal of discussion in the literature as to the reliability of measurements of $\Delta$ pH and $\Delta\psi$ (52, 94,.147). An idea of the reliability of this data can be attained by a comparison with values reported in the literature. These values are summarized in Table 22 (93, 146, 172, 173). To start with $\Delta\psi$ , earlier measurements reported for E. coli were mainly low (70, 75, 81 and 161 mV) and pH-invariant (Table 31), which was surprising since the $\Delta\psi$ would be expected to increase with pH if the PMF had to be kept constant. This pH-invariance has been ascribed to technical difficulties in measuring the $\Delta\psi$ (52). There are two more recent reports of the expected pH variability of $\Delta\psi$ , both of which give higher estimates of $\Delta\psi$ . Zilberstein et al. (218) quoted values of 102, 135 and 152 mV at pH 6, 7 and 8, respectively. Kashket (94) quoted values of 94, and 157 mV at pH 6.25 and 8.25. Both reports dealt with values determined in whole cells. The vesicle determinations reported here for the parent strain are in the same range and are therefore likely to be as valid as any obtainable with presently available techniques.

There is somewhat more agreement in the literature on the $\Delta$ pH values. This is probably because the $\Delta$ pH measurements are reproducible since they depend on an anion and there is no nonspecific binding of the probe. Values on cells at pHs from 5.5 to 6.25 varied from 95 to 118 mV (Table 31). At pHs around 7 the value of $\Delta$ pH in two cases were much lower than these values (35, 43mV) but in one case

Table 31

$\Delta\psi$, $\Delta$pH and PMF as a function of external pH in Escherichia coli as measured by different workers

| Measurements made on | External pH ($pH_o$) | $\Delta\psi$(mV) | $\Delta$pH(mV) | PMF(mV) | Comment | References |
|---|---|---|---|---|---|---|
| (1) Vesicles | 5.5 | 65 | 125 | 190 | $\Delta\psi$ almost constant | (173) |
| | 6.0 | 70 | 80 | 150 | | |
| | 7.8 | 75 | 0 | 75 | | |
| (2) Cells and Vesicles | 5.5 | 75 | 118 | 192 | $\Delta\psi$ constant | (172) |
| | 7.5 | 75 | 0 | 75 | | |
| | 8.0 | 75 | 0 | 75 | | |
| (3) Cells | 7.0 | 74 | 109 | 183 | $\Delta\psi$ almost constant | (146) |
| | 8.0 | 82 | 0 | 82 | | |
| | 9.0 | 88 | -30 | 58 | | |
| (4) Cells | 6.0 | 161 | 95 | 255 | $\Delta\psi$ constant | (93) |
| | 7.15 | 161 | 43 | 206 | | |
| (5) Cells | 6.0 | 102 | 105 | 207 | $\Delta\psi$ changes with $pH_o$ | (218) |
| | 7.0 | 135 | 35 | 170 | | |
| | 8.0 | 152 | -11 | 140 | | |
| (6) Cells | 6.25 | 94 | 100 | 194 | $\Delta\psi$ changed with $pH_o$ | (94) |
| | 8.25 | 157 | 0 | 157 | | |

it was in the same range (109 mV). The 109mV value comes from an early (1976), and therefore, likely to be technically less reliable, report from the same group that later reported a 35 mV value, and so probably should be discounted. Determinations at pH 7.5 and above gave values of 0 to -11 mV (Table 31).

A $\Delta$ pH value of 100 mV (94) corresponds to a $\Delta$ pH of 1.69 units. Thus, a determination of 100 mV at an external pH of 6.2 indicates an internal pH of 7.89 which is close to the 7.8 value expected for E. coli. Similarly, a value of 40 mV at pH 7 corresponds to a $\Delta$ pH of 0.67 units and an internal pH of 7.67. The $\Delta$ pH values in the literature therefore appear to be considerably more reliable than the $\Delta\psi$ values.

The value reported here for the vesicles from the parent strain at pH 5.5 was 124 mV which corresponds to 2.1 pH units. In the parent cells, at pH 6.6, the $\Delta$ pH was 58 mV, which corresponds to a 0.98 pH unit. These values are in the same range as the values quoted in the literature. Similarly, the $\Delta$pH in the cells at pH 8.2 is in agreement with the published values. In vesicles, the value of 29 mV at pH 7.8 is slightly high- it corresponds to 0.4 pH units. However, the $\Delta$pH determination becomes less reliable at and above 7.8 because now the $\Delta$ pH is in the reverse direction, and the concentration of the probe is higher outside. For this reason Padan measures the $\Delta$ pH at 7.8 and takes it as the zero $\Delta$pH value (220). It is clear then that the $\Delta$pH values reported here are in agreement with literature values. Therefore, the fact that the mutant and parent showed similar

values indicates that $\Delta$ pH is not affected in the cya mutant.

Usually the PMF values reported in the literature are not independent determinations, but are the summation of the values of $\Delta\psi$ and $\Delta$pH. Therefore, the PMF values are as variable as the $\Delta\psi$ values. It seems worthwhile therefore, to consider only the values where $\Delta\psi$ varies with pH. In one case, the PMF was quoted as 207, 170 and 140 mV at pH 6, 7 and 8, respectively (218). In the other case, the PMF was 194 mV at pH 6.25 and 160 mV at pH 8.25 (94). The PMF has also been calculated by measuring the steady state lactose accumulation ($\Delta\mu$lac) in a lac constitutive $\beta$-galactosidase negative mutant of E. coli. The PMF at pH 6 and 8, when measured from $\Delta\psi$ and $\Delta$pH values, were 207 mV and 138 mV (218). Corresponding $\Delta\mu$lac values were 162 and 142 mV. In another report by Kashket (94), the $\Delta\mu$lac values were essentially invariant with pH, 175, 169 and 163 mV, while the $\Delta\mu H^+$ (PMF measured by $\Delta\psi$ and $\Delta$pH) dropped from 194 to 160 and 127 mV. The values reported here for the parent strain were 251 mV for pH 5.5 and 181 mV for 7.8 in the vesicles, and 161 mV for pH 6.6 and 170 mv for pH 8.25 in the cells. These are slightly higher in the vesicles at pH 5.5, but probably not significantly different.

The fact that the values in the parent strain agree well with the literature values clearly strengthens the conclusion that the values in the mutant are significantly different. These determinations then, considered together with the transport data, indicate that $\Delta\psi$ is low in the cya mutant.

The immediately preceding discussion showed that the $\Delta\psi$ value

of the mutant is lower than that of the parent, and that one can have confidence in the experimental data. However, since the relation between PMF (and its components) and transport rates are not clearly understood, it is not yet clear that the mutant values are functionally lower than those of the parent. That is, the question still remains as to whether any given decrease in $\Delta\psi$ will actually result in a change in transport rate.

This question about the relationship between transport and the PMF, although technically difficult and unanswerable at present, is of importance in analyzing the data presented here. The PMF values for the vesicles from the parent strain were 251 mV at pH 5.5 and 181 mV at pH 7.8. However, the transport values at both these pHs were nearly the same. In mutant vesicles, both the transport values and the PMF were affected by pH. Moreover, the mutant PMF value at pH 5.5 was very close (171 mV) to the parent value at pH 7.8, yet the transport rate in the mutant at pH 5.5 was only 59% of that of the parent at pH 7.8. The value of the PMF for the mutant at pH 7.8 is clearly lower than that of the parent - 128 vs 181 mV. The literature supports the conclusion that transport is pH-invariant when the PMF (variant or not) is of normal magnitude. If one assumes that there is a threshold value of the PMF, below which active transport of solutes cannot be maintained at its usual rates, and took this value to be somewhere around 170 mV, one could account for the data reported here.

The PMF and the transport values for the cells from the parent strain were both pH-invariant. However, in the mutant strain the PMF

and the transport values both were influenced by the pH. What is surprising is that the PMF values at pH 8.25 was higher than the value at pH 6.6 but the transport value was lower at high pHs. Also, the PMF value at pH 7.8 in the mutant was not very different from that of the parent at pH 6.6, being 155 vs 161 mV, but the corresponding transport values were very different. It may be that the $\Delta\psi$ determinations are not reliable since many factors might have affected the values obtained (e.g., the non-specific binding, the concentration of the probe used and perhaps the wide range of the magnitude of $\Delta\psi$ that was measured). A measurement of $\Delta\mu$lac, which has been found to be less pH-influenced (94), may be a good approach to resolve this problem. There is, however, another difficulty with all PMF measurements, namely, that they are steady state measurements. The $\Delta\psi$ as measured gives information as to the electrical potential of the cells at any given time. It does not however indicate what problems the cell may actually have in setting up the $\Delta\psi$. The indicator transported is given in a very small amount and is not transported actively; its transport therefore does not cost the cell anything. But the amino acid transport costs energy, i.e. tends to dissipate the PMF. If the cell grows slowly because it has difficulty in setting up and/or maintaining a $\Delta\psi$, it may transport slowly for the same reason, but give a higher than otherwise expected $\Delta\psi$, which is a steady state determination.

Yet another explanation about the disagreement between the transport data and the PMF could be given if one considers the local

proton circuit model (97), since in that case the functionality of the bulk phase steady state $\Delta \mu H^+$ in performing the active transport would be questionable.

## Part 4. pH-Homeostasis Of Cytoplasm And Mechanism Of Resistance To Alkaline pHs In The $\Delta$cya Mutant.

From the point of view of pH control, E. coli has two constraints on its growth. The first is that it has to maintain a constant internal pH, presumably because that is the pH at which its components function properly. The second is that it derives energy by oxidizing substrates, and that this involves the extrusion, of protons as a necessary consequence of the spatial organization of its electron transport carriers. As a consequence of the proton extrusion the intracellular pH becomes alkaline. At an external pH of 7.8, this is a serious problem, which is circumvented by bringing the protons back into the cell (thus maintaining the internal pH constant) and extruding cations, (thus maintaining the electrical gradient). Among the cations, $Na^+$ and $K^+$ seem to play major roles.

Mutants lacking $Na^+/H^+$ (NHA) and $K^+/H^+$ (KHA) antiport activities have been isolated and were found to be unable to grow at alkaline external pH (166, 219, 220, 221). The NHA-deficient strain grew at doubling times similar to those of the parent, up to pH 8.0. At

higher pHs, the doubling time increased markedly, being about 5 times that of the parent at pH 8.5 (219), because of the problem in bringing in protons to keep the $pH_i$ at 7.8 (222). These mutants were isolated as being unable to transport melibiose and glutamate. These two compounds are transported by a PMF dependent mechanism, since their transport depends on the $Na^+$ gradient, and they are symported with $Na^+$.

The role of $Na^+/H^+$ antiport in maintaining the $pH_i$ is also seen in alkalophilic bacteria. These bacteria are able to grow at alkaline pH because of having a highly active $Na^+/H^+$ antiport which also makes them unable to grow at acidic pH because of the excessive acidification of the cytoplasm (105). A mutant of B. alcalophilus that is deficient in $Na^+/H^+$ antiport, is unable to grow at alkaline pHs, but it grows well at acidic pHs (105).

The $\Delta$ cya mutant, although it has difficulty in growing at high pHs, is not similar to the NHA-deficient mutant. The $\Delta$ cya mutant shows its deficiency in growth even at pH 6, and this deficiency increases with pH. Moreover, it transports glutamate just as well as it does proline; both are transported slowly and the transport rates become slower at high pHs with no difference between them. Since the NHA-deficient mutant shows its effect only at pHs above 7.8, and since the $\Delta$ cya mutant maintains its $pH_i$ at alkaline pHs and treats an amino acid transported with $H^+$ in the same way as the one which is transported with $Na^+$, the two mutants cannot have the same defect. In a recent report, it has been suggested that proline is transported

with $Na^+$ (192). If this is so, then the *cya* mutant may have a problem that affects the $Na^+$ gradient, such that both amino acids are transported inefficiently.

Mutants deficient in the KHA system were isolated by selecting for cells unable to grow at pH 8.3 (176). These mutants grew faster than the parent at pHs from 5.4 to 6.2, slower at pHs from 7 to 7.9, and they were unable to grow at pHs higher than 7.9. This is different from the $\Delta$ *cya* mutant. However, the KHA mutants are not described in enough detail to make a real comparison.

Both the NHA and the KHA mutants operate well at acidic pH. Therefore neither, or at most one, of these functions seems to be needed at acidic pHs. On the other hand, the fact that neither mutant grew well above pH 8, indicates that both exchangers are needed at high pH. It is known that potassium is required for maintaining a normal $\Delta\psi$ (12, 91, 92, 103). However, it is difficult to study the maintenance of $\Delta\psi$ in the absence of sodium, because sodium is a contaminant of all media. Nevertheless, the fact that neither mutant grows at high pHs seems to indicate that establishment of $\Delta\psi$ requires both functions, and likely both cations.

Skulachev (189, 190) has proposed that the $Na^+$ and the $K^+$ gradients play major roles in buffering the $\Delta$ pH and $\Delta\psi$, respectively. During growth the cell accumulates $K^+$ (thus maintaining a $K^+$ gradient with high $K^+$ concentration inside), and keeps $Na^+$ out (thus maintaining a $Na^+$ gradient with low $Na^+$ concentration inside). Both these processes require energy. At

starvation, both $K^+$ and $Na^+$ flow down their gradients, $K^+$ going through uniport, thus maintaining an electrical gradient, and $Na^+$ going through the $Na^+/H^+$ antiport, thus maintaining the $\Delta$ pH. A recent report provides evidence for this proposition of Skulachev (1). In this report, a β-galactosidase negative lactose-permease constitutive mutant was used to analyse the inhibition of growth due to the transport of lactose, with respect to $\Delta\psi$ and $\Delta$ pH, using media of different ion compositions. In a medium with approximately equal amounts of $Na^+$ and $K^+$ ( 80 mM each), lactose transport inhibited the growth rate at $pH_o$ 6.0. This inhibition was stronger at $pH_o$ 7.5. At both external pHs, the $\Delta\psi$ was decreased (more at a high pH), and the $pH_i$ was normal, a characteristic similar to that of the cya mutant. Cells growing in medium with high $K^+$ (162 mM) and minimal $Na^+$ (0.6-1.0 mM), stopped growing on transport of lactose at acidic pHs, because of the inability to maintain the $pH_i$. The normal growth as well as the $pH_i$ were restored by an addition of $Na^+$ (10-100 mM), indicating a role of $Na^+$ in maintaining the $\Delta$ pH. In medium with high $Na^+$ (162 mM) and minimal $K^+$ (20 mM), the transport of lactose decreased the growth rate and the $\Delta\psi$, indicating that an insufficient amount of $K^+$ in the growth medium affects the buffering of $\Delta\psi$. Since the cya mutant has no problem in maintaining the $pH_i$, the $Na^+$ involving system appears not to be affected, at least directly. The growth characteristics of the cya mutant seem to be similar to those of the lactose transporting cells growing in medium with enough $Na^+$ and insufficient $K^+$. Since the lactose transporting

cells growing in medium with enough $Na^+$ and low $K^+$, and the cya cells, both have low $\Delta\psi$, it may be that they are unable to accumulate a sufficient amount of $K^+$, inside the cell, in the former case because of the insufficient $K^+$ in the medium, in the latter case, perhaps due to the deficiency in the uptake system for $K^+$ and/or due to an alteration in its excretion. Ezzell and Dobrogosz (50) have reported that growth of a cya mutant is more sensitive to high concentrations of sodium chloride and potassium phosphate than the growth of its parent, and that this deficiency is reversed by adding cAMP. This is consistent with the present hypothesis of a decreased ability to carry out $K^+$ fluxes in the cya mutant. A high concentration of $K^+$ may be increasing the net uptake of $K^+$ by the cya cells, thus decreasing the $\Delta\psi$ even further. The sensitivity to the high $Na^+$ concentration may be due to the effect of $Na^+$ on the uptake of $K^+$, as has been reported by Sorensen and Rosen (196). The proposition that the $K^+$ fluxes are disturbed in the cya mutant can be verified by further studying the growth characteristics of cya mutant in media of various ion compositions with simultaneous determination of the $\Delta\psi$ and $\Delta$pH, as well as the $K^+$ concentration inside the cells. Since, according to Skulachev (189, 190) the $K^+$ gradient becomes important to buffer the $\Delta\psi$ at alkaline pH while the cytoplasm is being acidified, a deficiency influencing the $K^+$ gradient will be more obvious at alkaline pH - as observed in the cya mutant studied here.

Part 5. pH Shift Experiments.

The Δcya mutant responded to a shift in pH with a short lag in growth, whether the shift was from 6 to 7.8 or the reverse. Its parent was not noticeably affected. One might anticipate a difficulty when the pH is shifted upward, since at the higher pH the cell has to rely on a mechanism that brings protons in to keep the $pH_i$ constant and simultaneously has to maintain a $\Delta\psi$, and the mutant is defective in that. The NHA mutant in fact shows a long lag on transfer to high pH, before resuming growth at the slow rate characteristic of the new pH.

Padan and her colleagues have reported that the parent strain of the NHA mutant also shows a lag on transfer to pH 8.3, though this lag is very short (6 minutes)(220). The ΔpH was measured during this period, and dropped to zero immediately after transfer, and increased to 0.5 (inside acid) within 10 minutes. It seems then that the cell lost whatever ΔpH it had very quickly, and rebuilt the ΔpH in the opposite direction slowly. The re-establishment of the ΔpH did not require a new protein to be synthesized, as judged by the fact that it could take place in the presence of chloramphenicol.

Strain NA-1, the parent strain used here, did not show a lag on transfer in either direction. However, readings were taken only at 20 minute intervals, so that a very short lag could have been missed. The Δcya mutant had a longer lag, 60 minutes, than the NHA mutant—perhaps because it is deficient in development and/or maintenance of

electrical gradient.

Data for the pH-shift down of the NHA mutant and its parent were not given by Padan et al. (220). They reported however that "the mutant behaved normally after the pH jump from pH 7.2 to pH 6.4" (220). Since the data for the parent strain were not given, one cannot know whether both or neither had a short lag after transfer.

A shift down requires the establishment of a $\Delta$pH and a decrease in $\Delta\psi$. Suppose the normal strain loses its membrane potential on shift-down—perhaps losing both $\Delta$pH and $\Delta\psi$. Then, if the $\Delta$ cya mutant is slow in the development and/or maintenance of $\Delta\psi$, this would be enough to explain the lag.

Whatever the mechanism may be, the $\Delta$ cya mutant cannot cope immediately with a pH change in either direction. This is not reversed by the presence of cAMP. It seems then that a normal response to pH shift, like growth at high pH, depends on a product transcribed from a cAMP-CRP-controlled promoter, rather than on the mere presence of cAMP during the shift.

Part 6. Other Evidence That The cya Mutant Has A Lowered Membrane Potential: Evidence of changes in membrane function of the cya mutant.

The cya mutant has been reported to have an increased resistance to phage λ and $T_6$ due to a deficiency in the receptors (4, 108). It was also reported to be resistant to a large variety of agents including mutagens, antibiotics and sublethal heat and hypotonic shock (108). At the same time, it was reported to be more sensitive to neutral detergents, streptomycin and azide. This was all summarized as indicating fundamental alteration in the cell envelope (4, 108). But, according to a recent report, the translocation of several periplasmic and outer membrane proteins seems to require energy from the proton motive force (PMF) across the cytoplasmic membrane since the dissipation of PMF blocks the translocation of these proteins (175). Thus, structural defects seen in the cell envelope of cya mutants may be due to the defect in the energization of the membrane.

Absorption of colicins, and antibiotics like streptomycin, require energy as does the phage entry and the translocation of some of the periplasmic and outer membrane proteins. The defects seen in the cell envelope and membrane properties of the $\Delta$cya mutant therefore seem to be due to the decreased availability of the membrane energy. Strains which have decreased energization of their membrane, e.g. uncA (167, 168) and ecf/ssd (143) mutants, are reported to be

antibiotic resistant. The defects seen in the $\Delta$*cya* mutant therefore may be structural, due to some components in the cell envelope not being made, or functional, due to decreased availability of energy for transport, or both.

## Part 7. Some Speculations On The Nature Of The cya Defect.

The genetic evidence presented here is that the defect in the *cya* mutant results from a deficiency in transcription at a cAMP/CRP controlled system. This conclusion is based on the fact that the effect of the mutation can be overcome by a further mutation, which seems to be located in *crp*. This secondary mutation restores *crp* function in that the strain which carries both mutations is able to grow on lactose, maltose and arabinose. It seems likely therefore that it is the restored function of *crp* that restores pH - adaptability (resistance and ability to cope with the pH-shifts). Since *crp* functions in control of transcription, it would seem then that the defect in pH-adaptability of the $\Delta$*cya* mutant is a defect at the level of transcription.

The problem with this is that the pH-shift experiments described here are all carried out with glucose as the carbon source. In glucose-grown cells, the intracellular concentration of cAMP is very low. In continuous cultures limited with glucose (i.e., derepressed with respect to catabolite repression), internal cAMP was measured at

5.5 ± 0.7 μM (215). When ammonia was limiting and glucose in excess, this value dropped to 0.32 ± 0.02 μM. The same relation was seen in the values of extracellular cAMP. In a more recent report, the intracellular cAMP concentration was almost the same in glucose-limited cultures as in phosphate-limited (catabolite-repressed) cultures, around 1 μM in both cases (122). The The external concentration, however, varied greatly: around 0.4-1.0 μM in the glucose-limited cultures, and 0.05 μM in the phosphate-limited ones.

These relative concentrations can be looked at in another way. The cell volume in a late log culture makes up only 2-3 μl per ml of culture medium (211). Three μl at a 1 micromolar concentration actually contain much less cAMP than 1 ml at 0.05 μM. Thus, in repressed cells, about 95% of the cAMP found is outside the cell. The case of the derepressed cells is more astonishing- in that the extracellular value is about ten-fold higher; and the intracellular value does not change— indicating that over 99% of the cAMP is extracellular.

One should allow for the fact that cAMP determinations are notoriously difficult. Nonetheless, it is clear that most cAMP produced in E. coli is excreted, as is reviewed by Botsford (20, 122). However, it is widely accepted that the internal cAMP concentration of glucose-grown cultures is much lower than that of glycerol- or succinate-grown cultures. Moreover, the functional concentration must be lower since it is possible to induce

β-galactosidase in glucose-grown cells with IPTG if cAMP is not provided (148, 152).

In the presence of lactose and glucose, β-galactosidase is not synthesized. This is due to two effects- inducer exclusion by glucose which keeps lactose out, and the low level of cAMP. If the inducer exclusion is circumvented by adding IPTG, β-galactosidase can be made at 20% of the fully induced level (215). If cAMP is needed for this, as it clearly is, then there must be considerable amounts of both cAMP and CRP in glucose-grown cells.

It is not impossible, then, that transcription of various genes is cAMP/CRP dependent, even in glucose-grown cells. This could be nicely regulated if the relevant promoters have a higher affinity for cAMP/CRP than the promoters which are susceptible to catabolite repression.

There is evidence that promoters are affected by different cAMP concentrations. Induction of the arabinose operon of E. coli was shown to require more cAMP than induction of the lactose operon (111). On the basis of the different effects of a cAMP phosphodiesterase mutation on transcription from different operons, Alper and Ames also suggested that there is a "hierarchy" of cAMP-sensitive operons (6). It seems reasonable, then, that some operons may respond to very low levels of cAMP. However, a more plausible explanation can be given if one considers the role of cAMP as a negative effector in the transcription of several genes in E. coli (204). This is so because then one can consider E. coli having

two sets of genes, one set transcribed under conditions of catabolite repression (where cAMP acts as a negative effector), and the other transcribed under conditions when there is no catabolite repression (where cAMP acts as a positive controller). What is not so clear is the advantage to the cell in such a regulation.

If one considers the recent information on the cloned cya gene the topic becomes even more interesting. The cya gene seems to have three promotors (180, 204) and the active site of adenyl cyclase appears to be contained in a very small part of the enzyme molecule (109). Thus, it may be that the cya gene transcribes more than one gene product and that these different gene products are assigned to control different sets of cellular functions.

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