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The City University of New York, Ph.D., 1976
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THE UPTAKE AND UTILIZATION OF GLUCOSE 6-PHOSPHATE BY THE BLUE-GREEN ALGA, *ANABAENA FLOS-AQUAE*

by

PAUL MARC RUBIN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1976

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Several sugar phosphates were found to have a stimulatory effect on the rate of CO_2 fixation when they were supplied to photoautotrophically grown cells of *Anabaena flos-aquae* during a dark period prior to the start of the experiment. Maximal stimulation of the rate of CO_2 fixation under non preilluminating light conditions occurred with glyceraldehyde 3-P (80% at 0.75 mM), fructose 1,6 diP (200% at 5 mM), ribose 5-P (100% at 0.75 mM), and glucose 6-P (100% at 5 mM). Fructose 6-P slightly stimulated the rate of CO_2 fixation and 3-phosphoglycerate gave variable results. Except for the stimulation observed with glucose 6-P these results were qualitatively similar to those of Schacter *et al*. (1971) for isolated spinach chloroplasts. When these phosphorylated compounds were tested against cells that displayed nitrite inhibited CO_2 fixation only glyceraldehyde 3-P and glucose 6-P had any effect; both these compounds were found to increase the inhibition of CO_2 fixation caused by nitrite.

These results indicated that glucose 6-P might be assimilated by *Anabaena* 1444 as an intact molecule, and thus special attention was given to the study of the assimilation of this sugar phosphate. Using universally labeled ^{14}C-glucose 6-P, and ^{32}P-glucose 6-P it was found that both radioisotopes were assimilated by the

cells at the same rate and to the same extent, a finding that indicated that glucose 6-P was being assimilated as an intact molecule. This was confirmed by a chromatographic analysis of the cells which showed that the bulk of the radioisotope (80%) assimilated remained associated with glucose 6-P. Uptake experiments with universally labeled ^{14}C-glucose 6-P indicated that glucose 6-P was taken up to a much greater extent in the light (75 nmoles/mg chl·hr) than in the dark (30 nmoles/mg chl·hr). This increased uptake of glucose 6-P in the light was independent of the rate of turnover since Pelroy and Bassham (1972) showed that the oxidation of glucose 6-P by way of the pentose phosphate pathway (which is the major dissimilatory pathway for glucose 6-P in the blue-green algae) was inhibited in the light. The possibility did remain however that the increased uptake of glucose 6-P in the light was indicative of an energy dependent transport system. FCCP (an uncoupler) and Antimycin A (an inhibitor of cyclic photophosphorylation) were both found to be effective inhibitors of this photostimulated assimilation of glucose 6-P when compared to DCMU (an inhibitor of non-cyclic photophosphorylation). These findings strongly suggested that the light-mediated transport of glucose 6-P was energized by ATP derived mainly from the cyclic process.

The assimilated glucose 6-P could support limited metabolic work in the dark. Pretreatment of the cells with glucose 6-P significantly enhanced the rate of dark CO_2 fixation as compared to controls. This enhancement of dark CO_2 fixation was probably related to the alga's ability to oxidize the assimilated glucose 6-P in the dark and thus generate NADPH, and ribulose 5-P which could enter the Calvin cycle and enhance the rate of CO_2 fixation. The NADPH could also be coupled, to a limited extent to the NADPH oxidase system producing ATP, which could be utilized to stimulate CO_2 fixation.

It was found that the glucose 6-P which was assimilated by the cells in the dark was oxidized by way of the pentose phosphate pathway. In the light only 6.7 nmoles of glucose 6-P/mg protein·5 hr were respired as compared to 67 in the dark. This limited oxidation of glucose 6-P in the light agreed well with previous results of Pelroy and Bassham (1972). In the dark, there was a very limited turnover of glucose 6-P which was assimilated and this indicated a bottleneck in the aerobic respiratory pathway. Previous work (Grossman and McGowan, 1975) indicated that the enzymes of the pentose phosphate pathway were not limiting, thus implying that perhaps the NADPH oxidase system was the limiting factor. The dark uptake of glucose 6-P was then used as a probe for studying the oxidase system of these algae. It was found that various inhibitors of the

electron transport system of this alga such as SHAM, Antimycin A, and rotenone could inhibit the uptake of glucose 6-P by at least 60%. FCCP, oligomycin, and phloridizin also inhibited the uptake of glucose 6-P in the dark. These results indicated that the uptake of glucose 6-P in the dark was dependent on a functional and coupled oxidase system. The limited uptake of glucose 6-P in the dark was therefore related to the relatively inefficient nature of this oxidase system in producing ATP as compared to the photosynthetic production of ATP.

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I would also like to thank Ms. Ellen Zetoney for her excellent technical assistance during the course of this thesis investigation.

ABBREVIATIONS

DCMU	3-(3,4 DICHLOROPHENYL)-1,1 DIMETHYL UREA.
FCCP	CARBONYL CYANIDE p-TRIFLUOROMETHOXY-PHENYLHYDRAZONE.
SHAM	SALICYLHYDROXAMIC ACID
GLUCOSE 6-P	GLUCOSE 6-PHOSPHATE (ALL PHOSPHORYLATED COMPOUNDS ARE ABBREVIATED IN THE SAME FASHION).
HEPES	N-2 HYDROXYETHYLPIPERAZINE-N'-2-ETHANE SULFONIC ACID.
UL	UNIVERSALLY LABELED
TCA	TRICARBOXYLIC ACID CYCLE (KREBS CYCLE).
DSPD	DISALICYLIDENE PROPANEDIAMINE
DCIP	2,6-DICHLOROPHENOL INDOPHENOL
PMS	PHENAZINE METHOSULFATE

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The blue-green algae constitute an extremely interesting group of organisms with which one can study the evolution of various biochemical phenomena. These algae are unique among all photosynthetic organisms in that they perform the light reactions using two photosystems with chlorophyll _a_ as their main photosynthetic pigment while maintaining a prokaryotic cellular organization. Their prokaryotic organization closely resembles that of the bacteria in that a membrane bounded nucleus and other membrane bounded organelles are lacking. All other photosynthetic prokaryotes except the blue-green algae perform the photosynthetic light reactions using bacteriochorophyll _a_ instead of chlorophyll _a_ as the main photosynthetic pigment, and do not evolve O_2 from the splitting of H_2O by photosystem II. In these two respects the blue-green algae resemble the higher plants and eukaryotic algae.

In the higher plants and eukaryotic algae the chloroplast is the site of the photosynthetic reactions. The numerous similarities between the blue-green algae and the eukaryotic chloroplast prompted the early suggestions that the blue-green algae may have given rise to this organelle. There are two equally feasible hypotheses concerning the evolution of eukaryotic existence. The first is that a eukaryotic cell type could have arisen from a prokaryotic cell, such as one of the ancestral forms of the blue-green algae, by a partitioning of the various specialized regions

of the protoplasm (evolutionary theory, Uzell and Spolsky 1974), whereby an enclosing of the photosynthetic lamellae by either the outermost photosynthetic membrane or the plasma membrane would give rise endogenously by this hypothesis to a chloroplast-like organelle. The alternative theory, the endosymbiotic theory of organelle evolution, was first presented by Mereschkowsky in 1905 for chloroplast evolution, and by Wallin in 1920 for mitochondria. In this theory, the eukaryotic chloroplast was originally believed to have been a free living organism, probably an ancestral form of the modern blue-green algae. The free-living cyanophyte was ingested by a primitive aerobic eukaryote, and through subsequent evolutionary development became established as an endosymbiont. In support of the endosymbiotic theory the remarkable propensity of the blue-green algae to enter into symbiotic relationships is often pointed out. The cyanelles of *Glaucocystis*, which can be cultured separately after extraction from the host cell, have strong resemblances to the blue-green algae. These cyanelles are believed to have become highly modified during their endosymbiosis which often makes their identification difficult. It is tempting to think that the end result of such an evolutionary and developmental sequence might be a photosynthetic organelle incapable of independent growth (Marguilis, 1970; Fogg *et al*, 1973) *i.e.*, the chloroplast.

The numerous biochemical and physiological resemblances between the eukaryotic chloroplast and the blue-green algae have been put forth as strong support for the endosymbiotic theory of chloroplast evolution. This theory gained renewed support and importance when Chun (1963) showed that certain satelite DNA molecules can be localized in the chloroplast. The existence of extra-nuclear DNA localized in the chloroplast has also been confirmed by Sagan (1965) using autoradiography. It is now known that chloroplasts have a complete protein synthesizing mechanism involving species of tRNA and rRNA which are distinct from the cytoplasmic species, permitting at least some autonomy by this organelle. The chloroplast ribosomes are very similar in size to the blue-green algal ribosomes and are sensitive to the same inhibitors of protein synthesis (Carr and Craig, 1970), which are, however, ineffective in inhibiting cytoplasmic protein synthesis. Studies performed using inhibitors of protein synthesis specific for either the organelle or cytoplasmic system, and mutants of the 70S chloroplast ribosomes (Ac20) have shown a limited number of components to be manufactured in the chloroplast; among these are cytochrome 559, cytochrome Q and a component of the enzyme RuDP carboxylase (Sager, 1972). This limited autonomy has been offered as strong support of the endosymbiotic theory of organelle evolution. Most of this data, however, as Uzell and Spolsky (1974) point out, only allows

the endosymbiotic theory to possibly be true but the evidence does not prove the theory nor does it disprove the "evolutionary theory" of eukaryotic evolution.

Recently (Klein, 1970), the numerous similarities between the red algae, which are the most primitive of all the eukaryotic algal groups and the blue-green algae have been elucidated. Cohen (1973) believes that through further studies of the Rhodophyta's chloroplast the question of plastid evolution will be settled.

The blue-green algae are believed to be obligate photoautotrophs, since almost all of the blue-green algae which are grown successfully in the laboratory grew only when sufficient light and CO_2 were supplied. Very few of the forms grown were found to demonstrate any significant heterotrophic growth (Allen, 1966; Stanier _et al_, 1971; Baker and Bold, 1970). The actual biochemical basis for the obligate photoautotrophic nature of these prokaryotes is at best imperfectly understood.

In one of the early investigations into the biochemical basis of the obligate photoautotrophic mode of growth, Smith and co-workers (1967) were unable to detect several key enzymes in the intermediate carbon metabolism of these algae. The enzyme α-ketoglutarate dehydrogenase could not be detected in any of the crude preparations and only low levels of succinate and malate dehydrogenases were found.

This strongly suggested the possibility of an incomplete Krebs cycle in these algae. Using ^{14}C-acetate and ^{14}C-pyruvate, Smith et al. (1967) followed their incorporation into whole algal cells and found that only the glutamate family of amino acids was heavily labeled; neither compound donated significant label to asparate, threonine, lysine, or methionine. This confirmed the existence of an interruption of the TCA cycle at the α-ketoglutarate dehydrogenase step. This finding was later confirmed by Pearce, Leach, and Carr (1967) who found the TCA cycle was interrupted at the same point. They postulated that the TCA cycle in the blue-green algae is not used as an energy yielding pathway, but was utilized in a biosynthetic capacity to produce the porphyrins needed in chlorophyll biosynthesis.

The lack of a complete TCA cycle is not believed to be the ultimate cause for the obligate photoautotrophic existence of this group. The utilization of the cycle in a biosynthetic manner is perhaps a reflection of an obligate photoautrophic existence rather than the cause. The Krebs cycle is believed to have become relegated to a biosynthetic role after the algae were well on their way to becoming obligate photoautotrophs (Smith, 1967), by a modification of other aspects of their aerobic respiration.

In the blue-green algae, glucose has been shown to be oxidized by way of the pentose phosphate pathway with

the glycolytic pathway playing only a very limited role (Wildon and Rees, 1964; Cheung and Gibbs, 1968; Pelroy, Rippka and Stanier, 1972). This, therefore, ruled out the possibility of substrate level phosphorylation. The inability of Smith et al. (1967) to demonstrate an NADH oxidase system in these algae could therefore explain the biochemical basis for their obligate photoautotrophic nature. The inability of these algae to reoxidize NADH aerobically with concomitant ATP synthesis, due to the lack of such an NADH oxidase system, would therefore be reason in toto for these prokaryotic algae's inability to grow heterotrophically, since without an oxidative respiratory pathway coupled to ATP production they could not produce ATP except during photophosphorylation.

Horton (1968) reported that he was able to detect low amounts of NADH oxidase activity from particulate fractions of Anabaena variabilis and Anacystis nidulans; and in Leucothrix sp. he was able to detect a much larger quantity of NADH oxidase activity. Biggins (1969), studying in vivo changes in ATP, NADP, and NAD pool sizes as the algal cells were switched from aerobic to anaerobic conditions concluded that the oxidase system was probably NADPH dependent and at least partially coupled to ATP production. Biggins' findings were confirmed by the work of Leach and Carr (1970) on the electron transport system in Anabaena

variabilis. They found that the oxidase system in this alga was predominantly NADPH dependent, but was capable of utilizing NADH at lower rates. The oxidase system also appeared to be at least partially coupled, but seemed incapable of supporting active growth. It may, as Leach and Carr have described, provide for an "energy of maintenance" under non-photosynthetic conditions.

In Anabaena flos-aguae 1444 the electron transport system appears to be non-particulate and with a much higher affinity for NADPH than NADH (Goldberg, 1975). This system also appears to be partially coupled to ATP production and preliminary observations indicate that it is capable of supporting at least limited metabolic work. The belief that the blue-green algae are obligate photoautotrophs because they lack a coupled oxidase system is therefore an oversimplification, since these algae have been shown to have an oxidase system which appears to be at least partially coupled. The oxidase system as reported by Leach and Carr (1970) appears to be a relatively inefficient system; the exact nature of the deficiency is not understood at present (Goldberg, 1975), but may be related to the flavoproteins in the electron transport system.

In a survey of the ability of filamentous cyanophytes to grow heterotrophically, Khoja and Whitton (1971) reported that of twenty-four strains tested only seven failed to show any heterotrophic growth in the dark on 10 mM sucrose.

The data presented, however, did not give any information about the growth rates of these algae in the dark, except to indicate that they were much slower than in the light. The doubling times were often five times longer in the dark than in the light, and doubling times of as long as three months have been reported (Carr and Whitton, 1973). Dark growth is at best poor compared to photoautotrophic growth and the scattered observations of slow heterotrophic growth among this group do not detract from the generalization that these algae are photoautotrophic in nature. This generalization has been confirmed by Stanier (1971) in an extensive survey of the nutritional requirements of the unicellular blue-green algae. He found that none of the 40 strains examined showed detectable growth in the dark after one month on agar plates of minimal medium supplemented with either acetate, succinate, glutamate, pyruvate, or glucose. The control set of plates, which were incubated in the light, showed good growth. These data indicated that among the unicellular blue-green algae dark heterotrophic growth is relatively rare. Rippka (1972) tested thirty-eight axenic strains of unicellular blue-green algae for their ability to grow photoheterotrophically and chemoheterotrophically. Photoheterotrophic growth is the ability of photosynthetic organisms to increase their growth rate in the light in the presence of an energy rich organic compound such as glucose. Rippka (1972) devised a method using DCMU (10^{-5} M), an

inhibitor of non-cyclic electron flow, and glucose (1% w/v) to screen for photoheterotrophic growth of the various unicellular strains. Until Rippka's work there was no satisfactory test for photoheterotrophic growth among photosynthetic organisms. In the blue-green algae photoheterotrophic growth is best exhibited under conditions of low light intensity or low concentrations of atmospheric CO_2. She found that none of the strains were capable of growing in the light in the presence of DCMU and in the absence of glucose. If glucose was added to the medium in addition to DCMU, seven strains out of the thirty-eight tested showed a limited ability to grow photoheterotrophically, and four of these strains were also able to grow chemoheterotrophically.

In an investigation of glucose utilization in three unicellular blue-green algae, one of which was known to be capable of photoheterotrophic growth, it was found (Pelroy *et al.*, 1972) that the only strain able to metabolize the glucose significantly was *Aphanocapsa* 6714, which is the strain known to be capable of photoheterotrophic growth. Examining the three strains for the presence of the various enzymes involved in glucose metabolism, the two obligate photoautotrophic strains were found to have all the enzymes necessary for metabolizing the exogenously supplied glucose as did *Aphanocapsa* 6714. There is therefore no obvious reason from an enzymological point of view for the inability

of the obligate photoautotrophic strains to grow on and metabolize glucose. Pelroy et al. (1972) suggested that the two photoautotrophic strains lacked a specific glucose permease and therefore the lack of photoheterotrophic growth was due to the alga's inability to assimilate the exogenously supplied glucose.

In the light, glucose is not metabolized via the pentose phosphate pathway, which is the major dissimilatory pathway for glucose in these prokaryotic algae (Cheung and Gibbs, 1966). Pelroy and Bassham (1972) showed that the internal pool of glucose 6-P became very large in the light while the 6-phosphogluconate pool remained very small. Upon transferring the cells to the dark the glucose 6-P pool started to turn over by way of the pentose phosphate pathway and the 6-phosphogluconate pool increased concomitantly. The lack of turnover of the glucose 6-P pool in the light suggested that there was a mechanism to inhibit the turnover (Pelroy and Bassham, 1972). It was further suggested that RuDP formed during the operation of the Calvin Cycle in the light was capable of inhibiting the first enzyme of the pentose phosphate pathway, glucose 6-P dehydrogenase. Thus, in the light when the RuDP concentration would be relatively high, inhibition would occur and in the dark, when the RuDP concentration decreased, the enzyme would be released from inhibition and allow the turnover of hexose 6-P.

Grossman and McGowan (1975) did not find any inhibition by RuDP of the enzyme glucose 6-P dehydrogenase from extracts of *Anabaena* 1444 or *Anacystis* 625. However, ATP and NADPH were both found to be effective inhibitors of the enzyme. These two compounds could also effectively account for the lack of oxidation of glucose 6-P noted in the light, since the relative concentrations of ATP and NADPH would be the highest during photosynthesis and minimal in the dark.

These findings correlated with the work by Pelroy *et al*. (1972) with *Aphanocapsa* 6714. Measuring the turnover of exogenously supplied glucose by following the release of CO_2 both in the dark and in the light, Pelroy *et al*. found that only in the dark was there significant release of CO_2 from the C-1 position of glucose. This indicated that the oxidation of glucose by way of the pentose phosphate pathway occurred at significant rates only in the dark, and supported the idea that some product of photosynthesis inhibited the pentose phosphate pathway.

In strains capable of photoheterotrophic growth, in the presence of glucose either with DCMU or in low light, the sugar supplied was not viewed as being a source of energy for ATP formation but rather a source of reduced carbon skeletons. In the case of glucose this hexose could also be a source of reduced pyridine nucleotide to be used for biosynthetic purposes. During photoheterotrophic

growth ATP was viewed as being supplied primarily by cyclic phosphorylation. Biggins (1975) suggested that under low light conditions the relative amount of cyclic photophosphorylation increased, which would support the idea that it was the source of energy for photoheterotrophic growth.

Ingram _et al_. (1973) performed a detailed study of photoheterotrophic growth in the blue-green algae. They found that the action spectrum for photoheterotrophic growth closely resembled that of photosynthesis, with maximal stimulation of growth occuring in the region in which both chlorophyll and phycocyanin absorb. Measurements of stable carbon isotope ratios indicated that photoheterotrophic growth involved photosynthetic CO_2 fixation utilizing RuDP carboxylase of the Calvin cycle. This type of growth pattern appears to allow for at least a limited amount of nutritional versatility among certain blue-green algae. Under conditions of low light, limited CO_2 availability, and high concentrations of organic waste in the environment, certain blue-green algae will be stimulated to grow photoheterotrophically. From the results of Ingram _et al_. (1973), it would appear that the photoheterotrophic growth pattern of these algae involved a very efficient utilization of the organic carbon source. The organic substrate was assimilated and metabolized in the light, and any CO_2 respired was apparently refixed by the Calvin cycle; thus, there was a complete utilization of all the carbons of the assimilated compound.

Ingram *et al*. (1973) suggested that the term obligate photoautotroph, when it is used to describe the blue-green algae in general, be changed to obligate phototroph, this would denote the overwhelming light requirement for growth among the cyanophytes, but not restrict the carbon source requirement to CO_2 alone. As shown by Stanier (1971) and Rippka (1972), obligate photoautotrophy is the overwhelming nutritional mode among the unicellular blue-green algae. However, among the filamentous forms no systematic survey of photoheterotrophy has been performed and the ability to grow, at least to a limited extent, in the photoheterotrophic mode may be more prevalent than previously thought.

Recently the inflexibility in control of cyanophyte enzyme levels has become apparent and has been suggested as one of the basic reasons for their inability to grow chemoheterotrophically or photoheterotrophically. The lack of regulation in the level of production of enzymes, particularly those involved in autotrophic metabolism such as RuDP carboxylase, could provide an explanation for the strong preference for CO_2 exhibited by the blue-green algae when grown in mixotrophic conditions in the light. Pearce and Carr (1967) demonstrated the inability of these algae to respond to exogenous acetate in their growth media. They found that there was no change in the level of enzymes involved in acetate utilization in these algae whether

acetate was in the growth medium or not. This constancy in enzyme levels may indicate that the blue-green algae synthesize almost all of their enzymes at a constitutive level. This lack of change in enzyme level was also demonstrated in the case of glucose metabolism (Pearce and Carr, 1969), where it was found that none of the enzymes of the glycolytic pathway or the pentose phosphate pathway responded to exogenously supplied glucose in the growth media.

The lack of transcriptional regulation is believed to be one of the main causes for the inability of blue-green algae such as *Anabaena variabilis* and *Anacystis nidulans* to respond to environmental changes. The failure of organic compounds to stimulate the growth or respiration of most blue-green algae, despite incorporation into cellular constituents, seems to be the key characteristic of obligate autotrophic physiology. The inability of exogenously supplied compounds to stimulate heterotrophic growth is believed to be related to the lack of transcriptional control (Delaney, Dickson and Carr, 1973) demonstrated by these algae.

Several compounds have been found to be assimilated by the blue-green algae in the light. Acetate has been shown to be taken up at a much greater rate in the light than in the dark in *Anabaena flos-aquae* (Hoare *et al*., 1967; Tarrant, 1972). No increase in the alga's growth rate has been noted although appreciable amounts of the

acetate carbon are assimilated, even under limiting conditions. The uptake of ^{14}C-acetate by Anabaena flos-aquae (Tarrant, 1972) in the light resembled a carrier mediated uptake system with an apparent Km for acetate of 6.0 mM and a maximum velocity of uptake of 6 μmoles of acetate/gm.hr. In contrast to the light uptake, the dark uptake was directly proportional to the external acetate concentration between 5 and 100 mM. Hoare et al. (1967) found that the photo-assimilation of acetate was extremely sensitive to DCMU, a potent inhibitor of non-cyclic electron flow. This implied that the production of ATP and reductant from the photochemical reactions were involved in acetate assimilation in the light. Acetate is metabolized in the blue-green algae by way of the interrupted Krebs cycle, the glyoxylate shunt, and the urea cycle. No energy is generated and thus no growth will occur even though acetate is being assimilated and utilized in macromolecular biosynthesis.

Sugars are also known to be transported into blue-green algae in light mediated processes. Glucose (Pearce and Carr, 1969), sucrose (Fay, 1965), and fructose (Smith et al., 1967), are among the sugars which have been described as being transported in this manner. Under dim light conditions, glucose, (as described above) is an effective stimulator of photoheterotrophic growth in some species of blue-green algae. Biggins (1975) has suggested that under

dim light the process of cyclic photophosphorylation predominates and in addition to supplying the energy for cellular growth, may furnish the energy for enhanced sugar assimilation.

In the enterobacterium *E. coli*, a transport system has been described for aiding in the assimilation of various sugar phosphates, particularly glucose 6-P (Winkler, 1966; Fraenkel, 1964). This transport system has been found to be an inducible system with exogenous glucose 6-P acting as the sole inducer for it (Dietz, 1971; Winkler, 1970 and 1971; Pogell, 1966). The system is, however, capable of transporting other hexose phosphates including fructose 6-P, glucose 1-P, and mannose 6-P (Winkler, 1965; Dietz, 1971 III). The transport system is also known to be energy dependent since several known metabolic inhibitors such as azide, FCCP, and 2,4 dinitrophenol have been found capable of inhibiting the uptake of the sugar phosphates (Dietz, 1972; Winkler, 1973). In *E. coli* the uptake system for the sugar phosphates adds versatility to their heterotrophic metabolism. The cells, when placed in an enriched environment for glucose 6-P, are able to take up larger amounts of this energy rich substrate and greatly increase their growth rate (Winkler, 1965; Fraenkel, 1964).

In the blue-green algae no similar transport system has been described, and the possibility of glucose 6-P or any other sugar phosphate being assimilated as intact

molecules by a specific transport system would pose several interesting biochemical and evolutionary questions to the investigator. Since the blue-green algae are known to be photoautotrophic (see above discussion), the finding of a transport system for a high energy compound which does not increase their growth rate would certainly seem interesting from an evolutionary point of view. From the biochemical point of view the ability to promote uptake of a compound such as glucose 6-P would offer a very useful tool in studying the metabolism of these autotrophic algae since glucose 6-P is a key intermediate in the carbohydrate metabolism of the bluc-green algae. The control of glucose 6-P turnover *in vivo* could aid in obtaining some important clues concerning the mechanism of the obligate photoautotrophic physiology in these algae.

The work to be discussed in this thesis deals primarily with the assimilation and utilization of glucose 6-P. Emphasis was placed on the mode of incorporation of this hexose phosphate both in the light and dark in hopes of gaining some understanding of how an obligate photoautotroph, such as *Anabaena* 1444, assimilates an energy rich organic substance as glucose 6-P. The turnover and utilization of the exogenously supplied glucose 6-P was also examined in order to gain some understanding of the *in vivo* mechanisms controlling respiration in these algae in the light and dark. The long term aim was to try to understand

the biochemical and physiological basis of the obligate photoautotrophic nature of these algae.

Materials and Methods

A. Culturing and Harvesting of *Anabaena flos-aquae*.

Anabaena flos-aquae (1444) was grown in 1 liter of Bristol's medium plus soil extract (appendix A) in 2 liter cotton-stoppered Erlenmeyer flasks. The cultures were continuously shaken on a New Brunswick G-10 gyrotory shaker at 125 rpm in a controlled-temperature room at 23°. The algae were illuminated by a bank of Sylvania Gro-lux lamps, at a light intensity of 5×10^{5} ergs cm^{-2} sec^{-1} at the flasks' surface.

The cultures were monitored continuously for contamination by plating aliquots onto nutrient-agar plates and incubating the plates for 4 days at 23°. The cultures were judged to be axenic if after the 4 day incubation period the plates showed no bacterial or fungal growth. Only axenic cultures were used for experiments.

The cultures were harvested by centrifugation at 10,000 rpm (13,300 x gravity) for 20 minutes. The cells were then washed in fresh sterile media and resuspended either in Bristol's solution at pH 8.2 (for use in glucose-6-P uptake experiments) or 50 mM HEPES buffer at pH 8.0 (for use in CO_2 fixation experiments) to give a final chlorophyll concentration of 250 μgm/ml.

B. Chlorophyll Determination

Chlorophyll was determined by a modification of the method of Arnon (1949). One ml of the cell suspension

was added to 4 ml of 80% acetone and incubated at 49° for ten minutes. The suspension was then centrifuged and the absorbance of the supernatant solution was measured at 643 and 665 nanometers on a Gilford 240 spectrophotometer. The chlorophyll concentration was determined by using the following conversion:

$$A_{643} \text{ X } 202 + A_{663} \text{ X } 80.2 = \mu\text{gm of chl/ml.}$$

C. Carbon Dioxide Fixation.

Carbon dioxide fixation was followed in whole cells by measuring the incorporation of $H^{14}CO_3^-$ into acid-stable compounds. The radioactive assay contained 1 ml of a cell suspension in 50 mM Hepes at pH 8.0 (250 μgm chlorophyll), 0.5 ml of 25 mM HCO_3^- containing 8 μCi of $H^{14}CO_3^-$, and 0.5 ml of water. The assays were performed in 13 x 100 mm test tubes, with constant agitation provided by small Teflon-coated stir bars. The reaction tubes were maintained at 26° in a Plexiglas water bath and illumination of 10^7 ergs cm^{-2} sec^{-1} at the tube surface was provided by two banks of GE 75w reflector spot lamps.

At various time intervals 100 μl samples were withdrawn from the reaction mixture and the reaction terminated by mixing with 40 μl of 90% formic acid. A known volume of the acidified sample was pipetted onto a planchet, dried, and the radioactivity was counted in a Nuclear-Chicago gas flow planchet counter. All counts were corrected for the efficiency of the counter (20%, as determined by a radio-

active standard). The dark controls were performed in the manner described above, except that the reaction tubes were wrapped in aluminum foil.

D. Glucose 6-Phosphate Uptake.

The uptake of glucose 6-phosphate by intact cells was measured using uniformly labeled ^{14}C-glucose 6-phosphate, and determining the incorporation of radioisotope by the cells. The uptake assay consisted of 1 ml of cells suspended in sterile Bristol's medium, pH 8.2 (250 μgm chl/ml), 0.5 ml of 25 mM glucose 6-phosphate containing 0.8 μCi/μmole, and 0.5 ml of water. The assays were performed in 13 X 100 mm test tubes and continuously stirred by small Teflon-coated stir bars. The reactions were maintained at 28° in a Plexiglas water bath, and illumination of $5x10^6$ ergs cm^{-2} sec^{-1} was provided by a bank of 75w GE reflector spot lamps.

Samples were withdrawn at various intervals, washed twice in 6 ml of Bristol's solution (pH 8.2), resuspended in 3 ml of the same and filtered onto Whatman GF/C 2.4 cm glass fiber filters. The filters were then adhered to planchets and counted in a Nuclear-Chicago gas flow counter, and the counts corrected for the efficiency of the instrument (20%). Dark glucose 6-phosphate uptake was measured in the same manner except that the assays were performed in the dark.

E. Turnover of Exogenously Supplied Metabolites.

The oxidation of C-1 ^{14}C-glucose and (UL) ^{14}C-glucose-6-P was followed by trapping the respired carbon dioxide in 2N KOH. The assays were performed in 16 x 100 mm sidearm test tubes fitted with serum caps and a suspended center well containing fluted filter paper saturated with the 2N KOH. (The one ml incubation mixtures consisted of cells suspended in Bristol's solution and 25 μmoles of metabolite.) At the end of the incubation period 0.5 ml of 90% formic acid was introduced through the sidearm and 0.5 hr was allowed for CO_2 trapping to occur. The filter papers were removed from the wells and radioactivity was determined in a Beckman Model LS250 liquid scintillation system using a scintillation cocktail of Omnifluor (New England Nuclear), Triton X-100, and toluene (0.25:75:125: w:v:v). The reactions were performed either at room temperature or in a controlled temperature bath at 25°.

The experiments were performed either in the light or dark. For those assays which were carried out in the light, the light was supplied by GE 75w reflector spot lamps giving an intensity of 5×10^{6} ergs cm^{-2} sec^{-1} at the tubes' surface. The dark experimental tubes were wrapped in aluminum foil.

F. Chromatographic Procedure.

Incorporation of radioactive label into the cells was also analyzed by chromatography. The cells from the uptake assays were collected by centrifugation, washed twice in 6 ml of Bristol's solution at pH 8.2, repelleted, and lysed by the addition of 40 μl of concentrated formic acid. The cellular extract was then spotted on Whatman no. 1 chromatography paper and chromatographic separation was performed in a descending system for 15 hours, using Wood's GW_3 solvent (see appendix B). The chromatogram was dried and placed in contact with Kodak no-screen X-ray film in a cassette. The film was exposed to the chromatogram for 2-4 weeks after which time it was developed using Kodak liquid X-ray developer and fixer. The various spots from the ^{14}C-glucose 6-P and glucose 6-^{32}P chromatograms were eluted with water, lyophilized and redissolved in 0.1 ml H_2O. These were counted in a scintillation counter using the Omnifluor-Triton X-100-toluene cocktail.

G. Preparation of Glucose-6-^{32}P.

Glucose-6-^{32}P was prepared from glucose and labeled $AT^{32}P$ using hexokinase. The reaction mixture contained 0.1 ml of 100 mM Tris-HCl, pH 7.6, 0.01 ml of 100 mM glucose, 0.02 ml of purified yeast hexokinase, 0.01 ml γ-^{32}P-ATP, and 0.86 ml H_2O. The reaction was allowed to proceed at room temperature for 3-4 hours before being stopped by

quick freezing in a dry ice-acetone bath, followed by lyophilization and chromatography in Wood's GW_3 solvent. The radioactive spot corresponding to glucose 6-P was eluted from the chromatogram, lyophilized and rechromatographed in Wilkinson and Davies solvent (appendix C). The glucose-6-P spot was eluted, lyophilized and mixed with non-radioactive G6P for use in an uptake assay. 2-Deoxy-glucose-6-^{32}P was made by the above method with the exception that 2-deoxyglucose was substituted for glucose.

H. Identification of Chromatographic Spots.

The various standard and unknown phosphorylated compounds were identified by using the phosphate spray indicator of Ames (appendix D). After spraying with the indicator spray, the chromatogram was heated for 3 minutes at 85° and placed under a U.V. lamp to allow full color development.

I. Chemicals and Reagents.

All non-radioactive phosphorylated compounds used during this investigation were purchased from the Sigma Chemical Company. Glucose-6-P was purchased as the monosodium salt; fructose-6-P as the disodium salt; fructose 1,6 diP as the sodium salt, Sigma grade; ribose-5-P as the disodium salt; glyceraldehyde-3-P as the diethylacetal of the monobarium salt; glycerate-3-phosphate as the sodium salt grade 1; 6-phosphogluconate as the trisodium salt;

glucose-1-P as the disodium salt; and phosphoenolpyruvate as the mono potassium salt.

Universally labeled ^{14}C D-glucose 6-phosphate (specific activity of 200 mCi/mmole), D-glucose-1-^{14}C (specific activity of 45 mCi/mmole), and universally labeled ^{14}C-D-glucose-1-phosphate (specific activity of 262 mCi/mmole), were purchased from ICN Pharmaceuticals, Inc. D-glucose-1-^{14}C-6-phosphate (specific activity of 56.6 mCi/mmole), gamma labeled $AT^{32}P$ (500 μCi lots), and barium carbonate-^{14}C were purchased from New England Nuclear. The CO_2 was released from the barium salt in a closed system with an excess of 50% lactic acid, and then trapped in 0.2 ml of 3N NaOH, which was diluted to 1 ml before use.

The following inhibitors were generous gifts from Dr. M. Avron to Dr. R. McGowan: DCMU, FCCP and phloridizin. Antimycin A was purchased from Calbiochem, oligomycin and rotenone were purchased from the Sigma Chemical Company. All the above inhibitors were dissolved in 75% ethanol. SHAM, which was a generous gift of Dr. C. Bacchi, was dissolved in 50 mM Tris, pH 8.0.

Purified preparations of hexokinase and phosphoglucose isomerase were purchased from the Sigma Chemical Company. The hexokinase was Type C-130 from yeast, and the phosphoglucoisomerase was grade III purified from yeast.

A. The Effects of Calvin Cycle Intermediates on the Rate of CO_2 Fixation in *Anabaena flos-aquae* 1444.

In the blue-green algae the major pathway for autotrophic CO_2 fixation is by way of the "Calvin cycle" (Carr and Whitton, 1973). Dohler (1974) has recently found that in *Anacystis* CO_2 fixation could also occur by way of the proposed C-4 pathway with phosphoenolpyruvate carboxylase acting as the major CO_2 incorporating enzyme although this is not believed to be a major pathway of CO_2 reduction. Kandler (1961) determined the distribution of radioactivity among the compounds in the ethanol soluble fraction of *Anacystis nidulans* after exposure to $^{14}CO_2$ for varying periods of time, and found the labeling patterns to be the same as the labeling patterns of the green alga *Scenedesmus*. Kindel and Gibbs (1963) determined the distribution of radioactivity among the various carbon atoms of the hexose residues from the polysaccharide fraction of *Anacystis* following exposure to $^{14}CO_2$, and determined that the pattern of labeling found in *Anacystis* was the same as that found in the green alga *Chlorella*, which is known to fix CO_2 by way of the Calvin cycle. Norris *et al*. (1955) and Linko *et al*. (1957) found a different pattern, in which an appreciable percentage of the label entered the compound citrulline, indicating an operative urea cycle with some CO_2 fixation occurring during the synthesis of carbamyl phosphate from CO_2, NH_3, and ATP. Finally there also

appears to be an active photorespiratory pathway (Lex _et al._, 1971), which is active under conditions of high O_2 levels and low CO_2 concentrations. Lex _et al._ (1972) found that the rate of O_2 uptake in the light may be up to twenty times the dark rate and competed with the nitrogenase for photosynthetically produced reductant.

Schacter _et al._ (1971) examined the effects of the various phosphorylated intermediates on the rate of CO_2 fixation in isolated spinach chloroplasts and found that numerous compounds were able to stimulate the rate of CO_2 fixation. In the blue-green algae the major pathway for CO_2 fixation, as discussed, is also believed to be the Calvin cycle, but no study has ever been undertaken to examine the effects of the various intermediates of the cycle on the observed rate of CO_2 reduction. In Table 1 the effects of various phosphorylated intermediates of the Calvin cycle on the rate of CO_2 fixation in _Anabaena flos-aquae_ 1444 are shown. All the assays were performed under optimal pH and HCO_3^- conditions for CO_2 fixation in this blue-green alga (appendix E and F). Several compounds have been found to stimulate the rate of CO_2 fixation in this alga; glyceraldehyde-3-P, fructose-6-P, fructose 1, 6-diP, ribose-5-P, glucose-6-P, and phosphoenolpyruvate stimulate CO_2 fixation by at least 30% under conditions of a non preilluminating light regime. The maximal stimulation of CO_2 fixation is observed with

Table 1. The effect of phosphorylated metabolites on the rate of CO_2 fixation in *Anabaena flos-aquae*.*

Fixation of CO_2 was performed as described in Materials and Methods. In preillumination experiments the cells were illuminated for various periods of time prior to the introduction of metabolite and $NaH^{14}CO_3$ and the initiation of sampling.

Metabolite	Light Conditions	Effect	
		stimulation	inhibition
G-3-P	no pre-illumination	80% at 0.75 mM	60% at 2 mM
PGA	no pre-illumination	variable 0-8 mM	none
FDP	no preillumination	200% at 5 mM	none
	10 min. pre-illumination	none	50% at 5 mM
F-6-P	no pre-illumination	30% at 2 mM	30% at 7.5 mM
R-5-P	no pre-illumination	100% at 0.75 mM	none

TABLE 1. (CONTINUED)

METABOLITE	LIGHT CONDITIONS	EFFECT	
		STIMULATION	INHIBITION
G-6-P	NO PRE-ILLUMINATION	100% AT 5 MM	NONE
	4 MIN. PRE-ILLUMINATION	NONE	NONE
GLUCOSE	NO PRE-ILLUMINATION	NONE	NONE
PI	NO PRE-ILLUMINATION	100% AT 5 MM	NONE
	4 MIN. PRE-ILLUMINATION	23% AT 1.1 MM	100% AT 8 MM
PEP	NO PRE-ILLUMINATION	37% AT 2 MM	70% AT 8 MM

*CONTROL RATE, 120 MICROMOLES OF CO_2 FIXED/MG CHL. · HR

glyceraldehyde-3-P (80% at 0.75 mM), fructose 1,6-diP (200% at 5 mM), ribose-5-P (100% at 0.75 mM), and glucose 6-P (100% at 5 mM). Except for the stimulation observed with glucose 6-P these results were qualitatively similar to those of Schacter et al. (1971) for isolated spinach chloroplasts. In the isolated chloroplast system as in the blue-green algae, fructose 1,6-diP, ribose-5-P, and glyceraldehyde-3-P stimulated the rate of CO_2 to the maximal extent observed.

The stimulation of CO_2 fixation by exogenously supplied sugar phosphates was believed to be the result of the phosphates entering the isolated chloroplasts and filling the pools of the Calvin cycle which were depleted during the dark period prior to isolation of the chloroplasts. The incorporation of the exogenously supplied intermediates into the internal pools served to shorten the lag phase observed during CO_2 fixation with isolated chloroplast preparations (Walker, 1967). The lag phase has been shown by numerous workers (Walker, 1965; Backe et al., 1965) to be the result of a shortage of intermediates of the photosynthetic carbon reduction cycle in the chloroplasts. Laztko and Gibbs (1969) measured the changes of numerous intermediates of the Calvin cycle enzymatically and found that the lag phase is most closely associated with the levels of the pentose monophosphates. A similar lag in photosynthetic CO_2 fixation after a period of darkness is

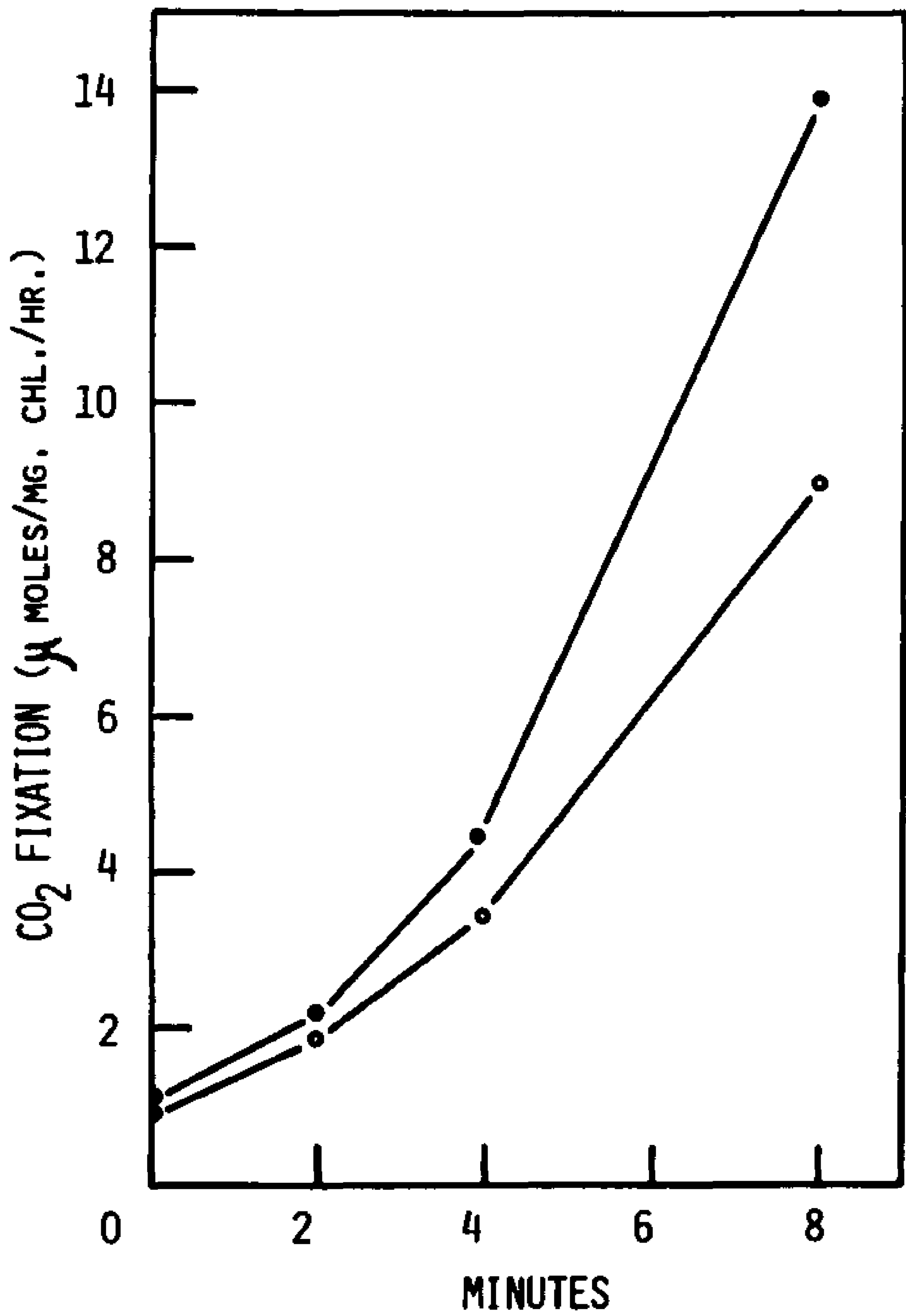
also a well known phenomenon in intact photosynthetic tissue (Gibbs, 1971). The duration of the lag in CO_2 fixation is a function of the duration of the dark period and the conditions to which the tissue has been subjected during this period. The stimulation observed with the phosphorylated intermediates after brief periods of darkness (10-15 minutes) shown in Table 1, can be attributed partially to the exogenously supplied intermediates shortening the lag phase for photosynthesis, and to an increase in the maximal rate of CO_2 fixation (Figure 1). One of the mechanisms by which these phosphorylated intermediates may increase the maximal rate of CO_2 fixation may be by acting as stabilizers or activators of a component needed for maximal CO_2 fixation to occur, as was noted in the isolated chloroplast system.

In the chloroplast system no stimulation or only very limited stimulation was observed with hexose 6-phosphates, such as fructose 6-P and glucose 6-P (Schacter, 1971). This was presumed to be due to a general impermeability of the chloroplast envelope to the hexose 6-phosphates (Schacter et al., 1971; Gibbs, 1971; Bamberger and Gibbs, 1965). In Anabaena 1444 only a slight stimulation in the rate of CO_2 fixation by fructose 6-P was observed (30% at 2 mM); however, a large increase in CO_2 fixation was observed when the cells were pretreated with glucose

Figure 1. The effect of glucose 6-P on the rate of CO_2 fixation by *Anabaena flos-aquae*. The CO_2 fixation was performed as described in Materials and Methods section C.

CONTROL (○)

2.5 mM GLUCOSE 6-P (●)

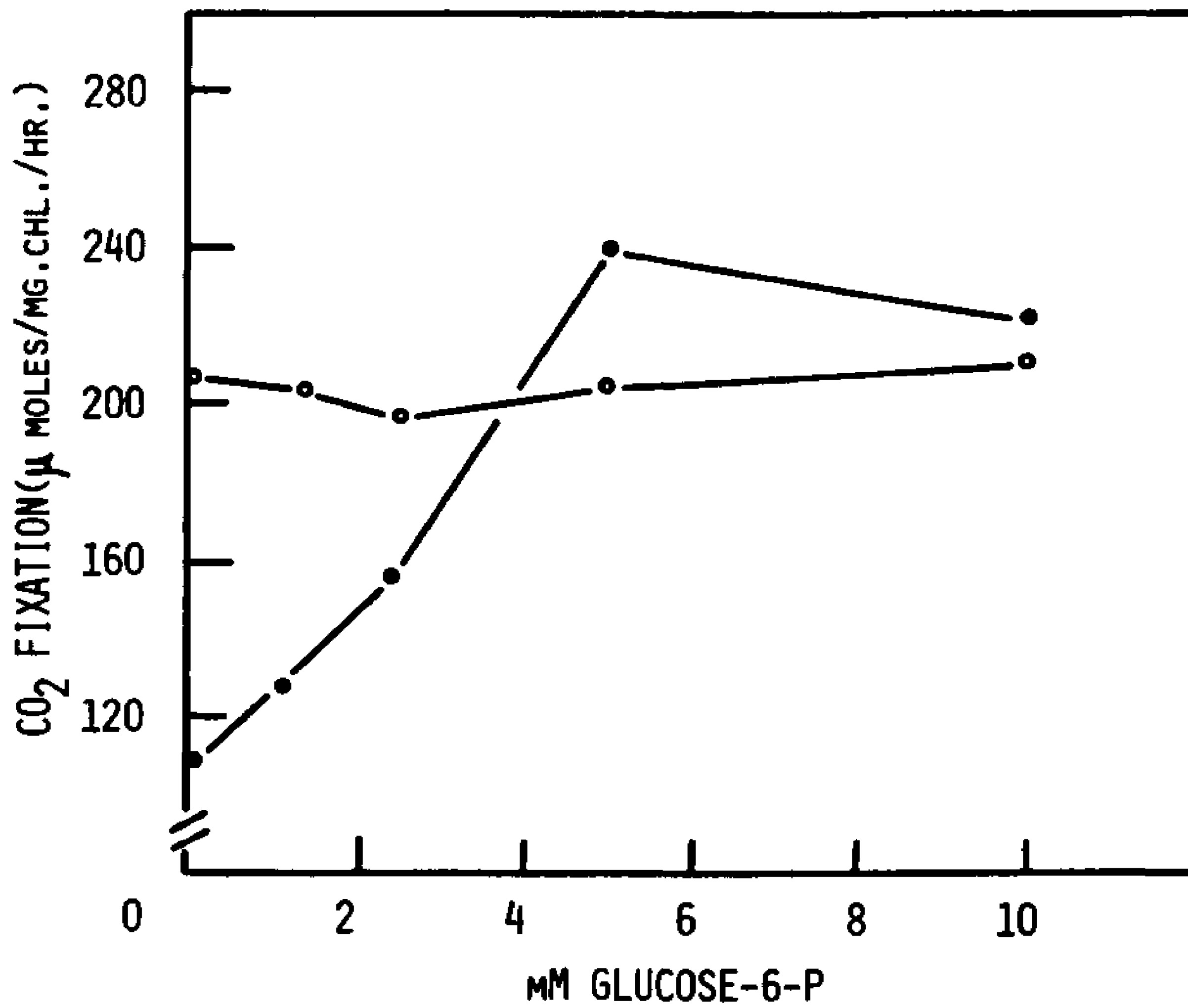


CO2 FIXATION (μ MOLES/MG. CHL./HR.)
0
2
4
6
8
10
12
14
MINUTES

6-P (100% at 5 mM) under a light regime with no preillumination. Glucose has been found to have no effect on the rate of CO_2 fixation but Pi does have a very significant stimulatory effect on the rate of CO_2 fixation (100% at 5 mM). This stimulatory effect observed with Pi on the rate of CO_2 fixation is at a much higher concentration of Pi (5 mM) than would be predicted to be stimulatory based on the studies with isolated chloroplasts (O'Neal *et al.*, 1972; Cockburn *et al.*, 1968). In isolated corn chloroplasts (O'Neal *et al.*, 1972) Pi stimulates the rate of CO_2 fixation by up to 80% at concentrations under 0.25 mM and becomes strongly inhibitory at concentrations above 2.5 mM.

The effects observed with the phosphorylated intermediates on the rate of CO_2 fixation in *Anabaena* 1444 after a short preillumination were significantly different from the effects observed when the cells were given a non preilluminating light regime. Fructose 1,6 diP inhibited the rate of CO_2 fixation after a ten minute preillumination, while Pi only slightly stimulated the rate of CO_2 fixation at concentrations up to 1 mM, and became inhibitory at higher concentrations. The effects observed with glucose 6-P on the rate of CO_2 fixation are shown in Figure 2. From these data it was observed that after a four minute preillumination, glucose 6-P had no effect on the rate of CO_2 fixation over a concentration range of 0-10 mM. Pelroy and

Figure 2. The effect of preillumination on the rate of glucose 6-P stimulated CO_2 fixation in *Anabaena flos-aquae*. The CO_2 fixation was performed as described in the Materials and Methods section C. Cells were illuminated for 4 minutes prior to the addition of $NaH^{14}CO_3$ and glucose 6-P (○), or cells were maintained in darkness until the addition of $NaH^{14}CO_3$ (●).


280
240
200
160
120
0
2
4
6
8
10
CO_2 FIXATION(μ MOLES/MG.CHL./HR.)
mM GLUCOSE-6-P

Bassham (1972) have shown that in the blue-green algae, glucose 6-P is not metabolized in the light; the internal pool of glucose 6-P increases and becomes almost saturated within four minutes. The level of 6-phosphogluconate, the product of glucose 6-P oxidation by way of the pentose phosphate pathway, remains low during the light period. Upon transferring the cells to the dark the glucose 6-P pool decreases very rapidly with a concomitant increase in the 6-phosphogluconate pool. This may be indicative of a regulation of glucose 6-P oxidation in the light, perhaps operating via the pentose phosphate pathway, which is known to be the major dissimilatory pathway for glucose 6-P in the blue-green algae (Cheung and Gibbs, 1966). Grossman and McGowan (1975) have shown this control to be exerted at the level of the first enzyme of the pentose phosphate pathway, glucose 6-P dehydrogenase. They found that ATP and NADPH, both products of the photosynthetic light reactions, were feedback inhibitors of this enzyme. Therefore, a four minute preillumination of the cells should maximize the internal pool of glucose 6-P, since its turnover would be prevented by the feedback inhibition. Exogenously supplied glucose 6-P thus should have little or no effect on the rate of CO_2 fixation when it is supplied to these preilluminated cells. On the other hand, if the glucose 6-P were supplied to the cells in the dark period, the internal glucose 6-P pool will be minimal and the exogenously

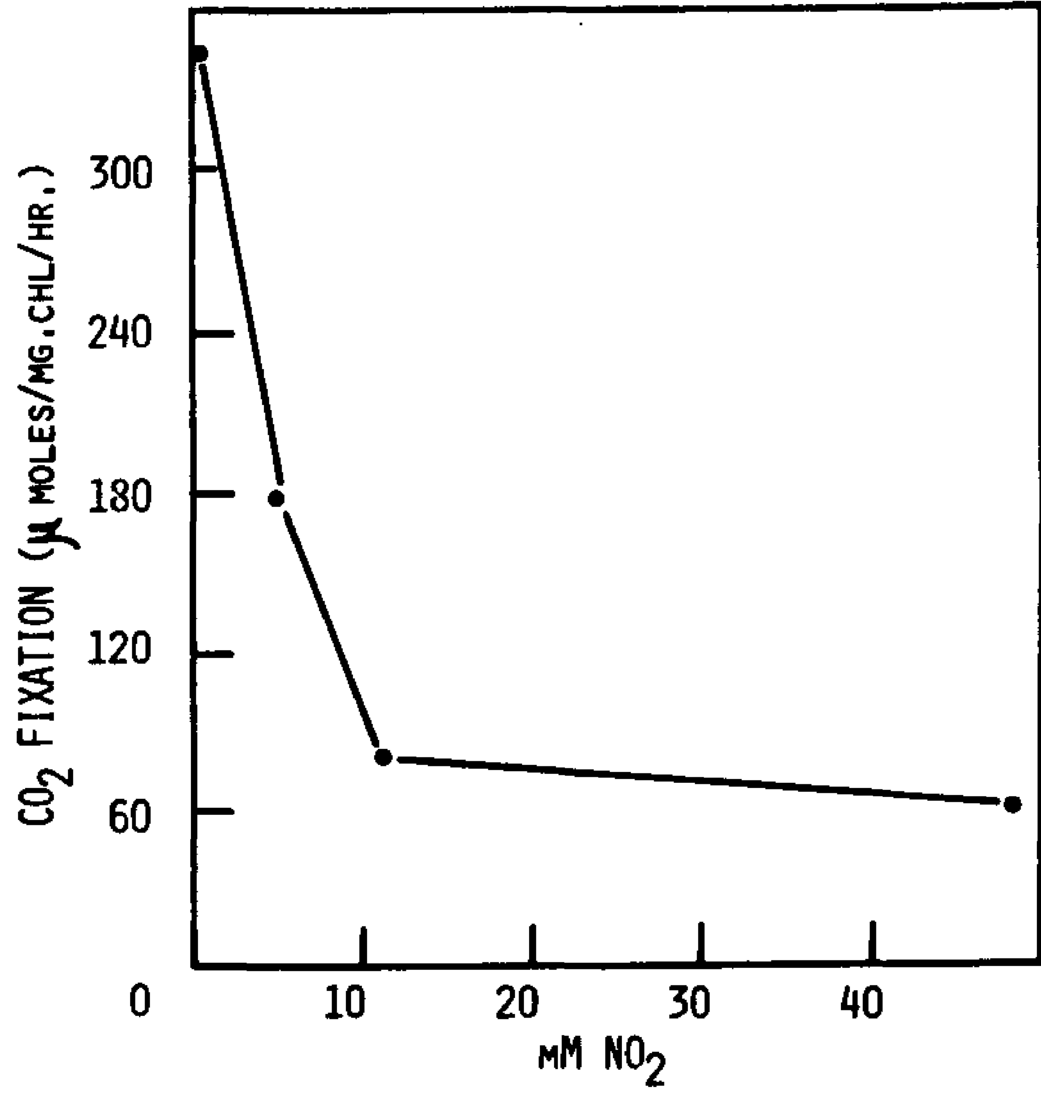
supplied glucose 6-P would be oxidized by way of the pentose phosphate pathway, which would supply NADPH and ribulose-5-P to the Calvin cycle and stimulate the rate of CO_2 fixation.

Phosphoenolpyruvate stimulated CO_2 fixation by 37% at 2 mM and became inhibitory at higher concentrations. This stimulation by phosphoenolpyruvate was predictable based on the findings of Jansz and McClean (1973), and Dohler (1974), who found that there was a minor pathway of CO_2 fixation in the blue-green algae which involved the carboxylation of phosphoenolpyruvate. Phosphoglycerate was found to give variable results on the rate of CO_2 fixation when tested over a wide concentration range, which is also similar to the results found for isolated chloroplast systems (Schacter *et al*., 1971).

B. The Effects of Calvin Cycle Intermediates on the Rate of Nitrite Inhibited CO_2 Fixation.

Nitrite is known to be reduced to NH_3 using NADPH as the *in vivo* H-donor (Hattori and Meyers, 1966). Hattori (1962) found that low concentrations of nitrite inhibited the rate of CO_2 fixation under conditions of low light intensity (600 lux) but had no effect under conditions of higher light intensities (10,000 lux). The effects of nitrite on CO_2 fixation under conditions of high light intensity are shown in Figure 3; the inhibition curve presented with *Anabaena* 1444 could be duplicated with

Figure 3. The inhibition by KNO_2 of the rate of CO_2 fixation by cells of *Anabaena flos-aquae*. Fixation of CO_2 was performed and measured as described in section C of the Materials and Methods.


CO2 FIXATION (μ MOLES/MG.CHL/HR.)
0
60
120
180
240
300
10
20
30
40
mM NO2

Anacystis 625. Maximal inhibition occurred at 15 mM nitrite, and this was the concentration used in all the inhibition studies on CO_2 fixation.

Nitrite inhibition of CO_2 fixation can be viewed as being the result of two distinct processes: one a competition between nitrite reductase reaction and the glyceraldehyde 3-P dehydrogenase reaction of the Calvin cycle for NADPH; and the second, the reduction of nitrite to NH_3 which has the capacity to act as an uncoupler of photophosphorylation, and/or to drain carbon skeletons from the Calvin cycle by stimulating their transamination to amino acids. The reduction of nitrite in higher plants has been shown to require two enzyme systems (Kessler and Zumft, 1973; Ramirez *et al*., 1965), an NADPH reductase and nitrite reductase. Ferredoxin has been shown to be the electron carrier mediating the transfer of electrons from the NADPH reductase to the nitrite reductase complex. Hattori and Meyers (1966) showed that nitrite reduction is NADPH specific in *Anabaena*, which was similar to most higher plants, but was unlike the green alga *Ankistrodesmis* in which the reductase has been shown to have an equal affinity for NADH and NADPH. The K_M for NADPH of the nitrite reductase complex in *Anabaena* is 1×10^{-5} M which is a lower Michaelis constant than reported for most other organisms; this would make the enzyme a good competitor with other NADPH requiring systems in the blue-green algae.

Table II shows the effects that the various phosphorylated intermediates had on the rate of nitrite inhibited CO_2 fixation. Phosphoglycerate, ribose-5-P, fructose-6-P and Pi, as shown in this table, were all without effect. Glyceraldehyde-3-P and glucose-6-P were found to enhance the observed inhibition of CO_2 fixation caused by nitrite. The increase in the nitrite inhibition of CO_2 fixation caused by glyceraldehyde-3-P was apparently related to their capacity to generate reduced pyridine nucleotide (NADPH). It has become increasingly clear in the past few years that the regulation of carbohydrate and amino acid metabolism in the blue-green algae is intimately associated with the relative endogenous pool sizes of NADPH and NADH (Batt and Brown, 1974; Grossman and McGowan, 1975). Batt and Brown (1974) demonstrated close correlations between NADPH consuming activities, such as the reduction of nitrite to ammonia, and the presence of increased levels of the pentose phosphate pathway. This was also shown in higher plants by Butt and Beevers (1961), who demonstrated that nitrite reduction stimulated the activity of the pentose phosphate pathway relative to the glycolytic pathway. The increased amount of reduced pyridine nucleotide produced by the oxidation of exogenously supplied glucose 6-P and glyceraldehyde 3-P was viewed as being shunted to the nitrite reductase complex where it was reoxidized in the process of nitrite reduction to ammonia. It was this increase in

TABLE II. THE EFFECT OF PHOSPHORYLATED METABOLITES ON THE RATE OF NITRITE INHIBITED CO_2 FIXATION IN *ANABAENA FLOS-AQUAE*.

THE EXPERIMENTAL PROCEDURES WERE THE SAME AS THOSE IN TABLE I WITH THE EXCEPTION THAT EACH ASSAY HAD 15 mM IN KNO_2.

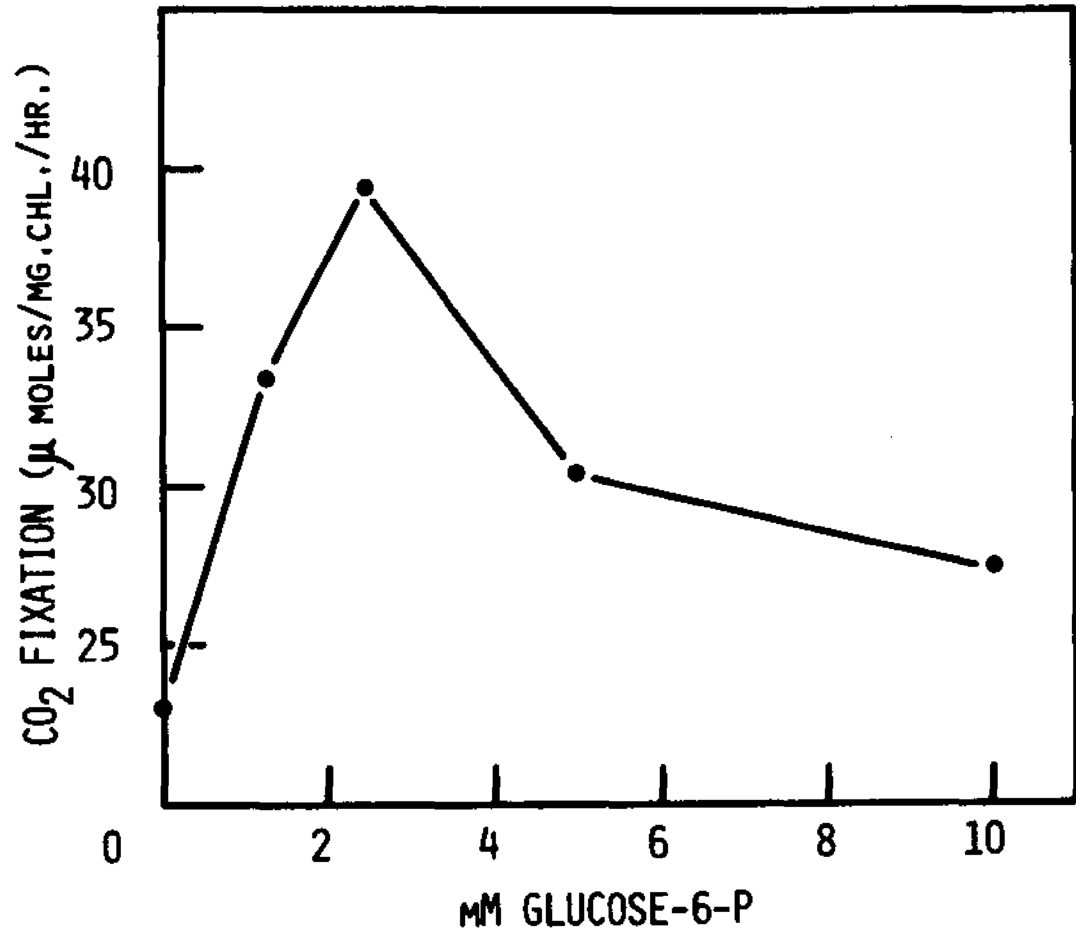
METABOLITES	LIGHT CONDITION	EFFECT ON NO_2 INHIBITION
G-3-P	NO PRE-ILLUMINATION	INCREASED 75% AT 4 mM
PGA	NO PRE-ILLUMINATION	NONE AT 0-10 mM
R-5-P	NO PRE-ILLUMINATION	NONE AT 0-5 mM
F-6-P	NO PRE-ILLUMINATION	NONE AT 0-10 mM
G-6-P	NO PRE-ILLUMINATION	INCREASED 66% AT 7.5 mM
	4 MIN PRE-ILLUMINATION	INCREASED 47% AT 10 mM
GLUCOSE	NO PRE-ILLUMINATION	NONE AT 0-10 mM
PI	NO PRE-ILLUMINATION	NONE AT 0-10 mM

the vegetative cells' ammonia pool which was believed to be responsible for the increased inhibition of CO_2 fixation. The ammonia as mentioned can act as an uncoupler of photophosphorylation and/or by displacing carbon skeletons from the Calvin cycle to produce amino acids.

If the cells were preilluminated for four minutes before the start of the experiment the exogenously supplied glucose 6-P was still effective in increasing the nitrite inhibition of CO_2 fixation. There was a 47% increase in inhibition caused by glucose 6-P after a four minute preillumination as opposed to a 66% increase in the nonpreilluminated experiments. The presence of nitrite seemed to prevent the complete inhibition of the pentose phosphate pathway. It would appear that in these experiments nitrite was acting both as a stimulator of the pentose phosphate pathway activity and as an effector of the inhibition of CO_2 fixation.

DCMU, an inhibitor of non-cyclic electron transport, should allow at least limited formation of ATP from cyclic photophosphorylation, but should prevent NADPH production since this is a non-cyclic process. Since CO_2 fixation by way of the Calvin cycle requires both NADPH and ATP, DCMU is a potent inhibitor of this process. Exogenously supplied glucose 6-P increased the inhibition of nitrite inhibited CO_2 fixation, glucose 6-P should theoretically be able to stimulate at least to a limited

Figure 4. The effect of glucose 6-P on the rate of DCMU inhibited CO_2 fixation by *Anabaena flos-aquae*. The CO_2 fixation was performed as described in the Materials and Methods section C. The cells were inhibited with 0.5 μM DCMU.



CO_2 FIXATION (μ MOLES/MG.CHL./HR.)
40
35
30
25
0
2
4
6
8
10
mM GLUCOSE-6-P

extent DCMU inhibited CO_2 fixation. Since DCMU reduces the internal concentrations of NADPH and ATP, glucose 6-P dehydrogenase should be released from full inhibition in the light; exogenously supplied glucose-6-P therefore should be oxidized generating NADPH which could feed into the Calvin cycle to help to stimulate the rate of CO_2 fixation. In Figure 4 the stimulation by exogenously supplied glucose-6-P of DCMU inhibited CO_2 fixation is shown. The results presented with nitrite and DCMU demonstrate the ability of the exogenously supplied glucose 6-P to generate reduced pyridine nucleotide which can be utilized by the various reductive enzyme systems (nitrite reductase, Calvin cycle).

C. The Demonstration of the Intact Assimilation of Glucose 6-P

The effects observed with the various phosphorylated compounds, particularly glucose 6-P, were of great interest since it was generally believed that phosphorylated compounds particularly the hexose monophosphates did not enter the cells intact (Schacter, 1971; Lichenstein _et al_., 1960). The experiments discussed above indicated the possibility that the phosphorylated compounds were entering the cells as intact molecules to produce the observed effects. In Figure 5 the rates of assimilation of glucose 6-P in both the light and dark are shown under optimal pH and temperature conditions (appendix G). The rate of glu-

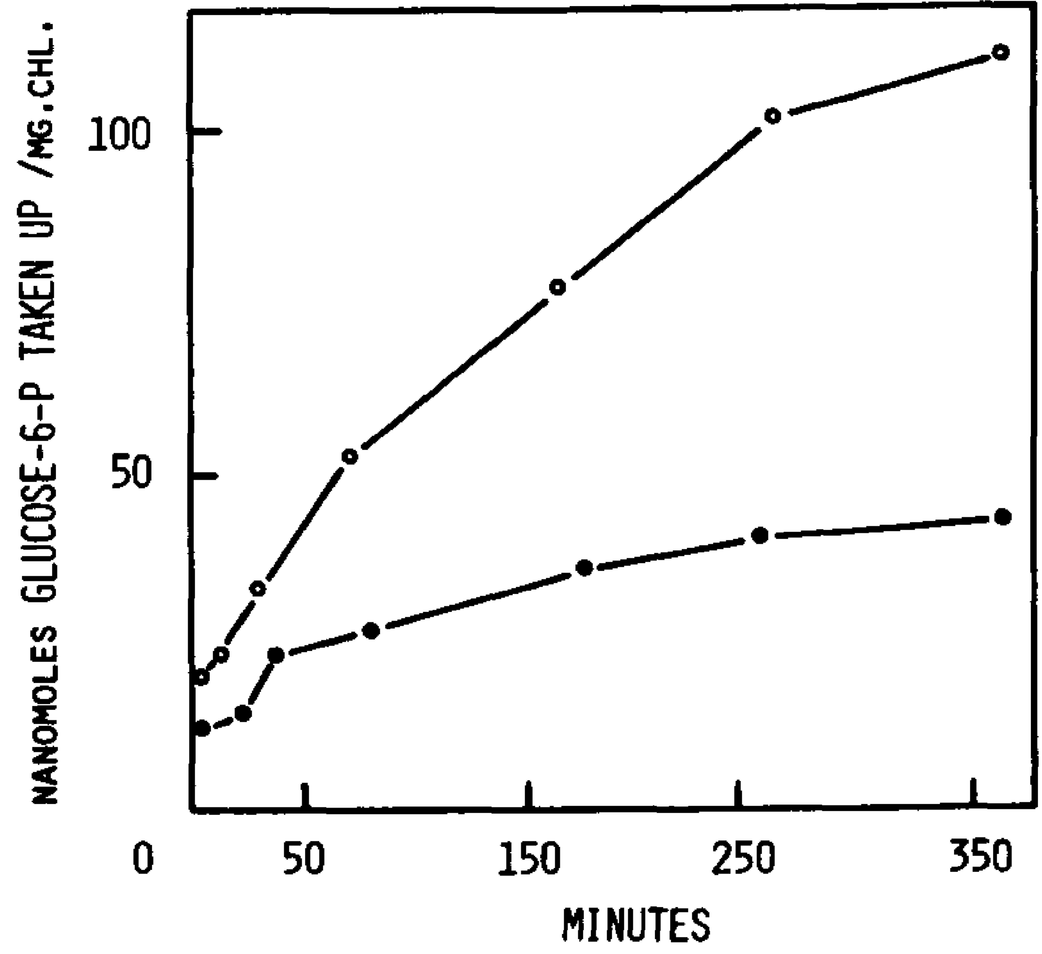
cose 6-P assimilation in the light was much greater than in the dark; in the light the rate of uptake was usually about 75 nmoles/mg chl. hr, and in the dark the rate was 30 nmoles/mg chl. hr. The data shown in Figure 5 was recorded over a six hour period; however, all subsequent uptake studies were performed over a 90 minute period with the rate of uptake calculated from the linear portion of the curve between 0-30 minutes. To eliminate the possibility of non-specific binding of glucose-6-P to the cells, an uptake experiment was performed in which the cells were pelleted, washed twice, and resuspended in a small volume of fresh medium. The cells were then broken in an Eaton press, the cellular debris separated from the supernatant solution by centrifugation, and the radioactivity associated with the debris and supernatant solution measured. Over 85% of the radioactivity was associated with the supernatant solution; the particulate fraction contained the remainder. A chromatographic analysis of the two fractions confirmed that essentially all of the radioactivity was in the supernatant solution with no detectable radioactivity in the debris. These results indicated that the radioactivity was entering the cells and not just adhering in a non-specific manner to the outer cell wall (gelatinous sheath), or membrane.

The measurement of glucose 6-P assimilation by intact cells of _Anabaena flos-aquae_ 1444 using (UL)-^{14}C-

Figure 5. The assimilation of glucose 6-P by Anabaena flos-aquae. Uptake of glucose 6-P by the cells was determinted as described in the Materials and Methods section D. The incubation mixtures contained cells equivalent to 250 μg chlorophyll and 6.3 μmoles glucose 6-P (specific radioactivity 0.16 μCi per μmole).

Assimilation in the light (○)

Assimilation in the dark (●)


NANOMOLES GLUCOSE-6-P TAKEN UP /MG.CHL.
100
50
0
50
150
250
350
MINUTES

glucose 6-P can only indicate whether or not the hexose sugar is entering the cells and not whether the intact sugar phosphate is being assimilated. To ascertain whether the hexose phosphate is assimilated as an intact molecule it is necessary to study the uptake of the sugar phosphate using several different approaches.

A comparison of the uptake of ^{14}C-glucose in the light and dark was performed with intact cells of _Anabaena_ 1444. As shown in Figure 6, there was only a slight difference between the rate of uptake of glucose accumulation in the light and dark (27%) as opposed to a 2.5 fold difference in the rate of glucose 6-P uptake in the light versus the dark noted earlier. The difference in the rates and extent of assimilation of glucose and glucose 6-P in the light and dark indicates a difference in the mechanism of transport for the two compounds.

The assimilation of glucose 6-P in the light by _Anabaena_ 1444 was challenged by high concentrations of ^{12}C-glucose. As shown in Figure 7, over a concentration range of 0-24 mM glucose, the assimilation of glucose 6-P was unimpaired. This lack of any substantial inhibition by glucose on glucose 6-P uptake can be interpreted as indicating that the uptake of glucose 6-P by intact cells of _Anabaena flos-aquae_ 1444 does not involve a dephosphorylation at the cells' surface. If there were a dephosphorylation at the cells' surface with the release of ^{14}C-glucose

Figure 6. The assimilation of ^{14}C-glucose in the light and dark by *Anabaena flos-aquae*. The assimilation of glucose was followed as described in the Materials and Methods section D. The 2 ml incubation mixture contained cells equivalent to 250 μg chlorophyll, and 6.3 μmoles of glucose (specific radioactivity 0.16 μCi per μmole).

Assimilation in the light (●)

Assimilation in the dark (○)

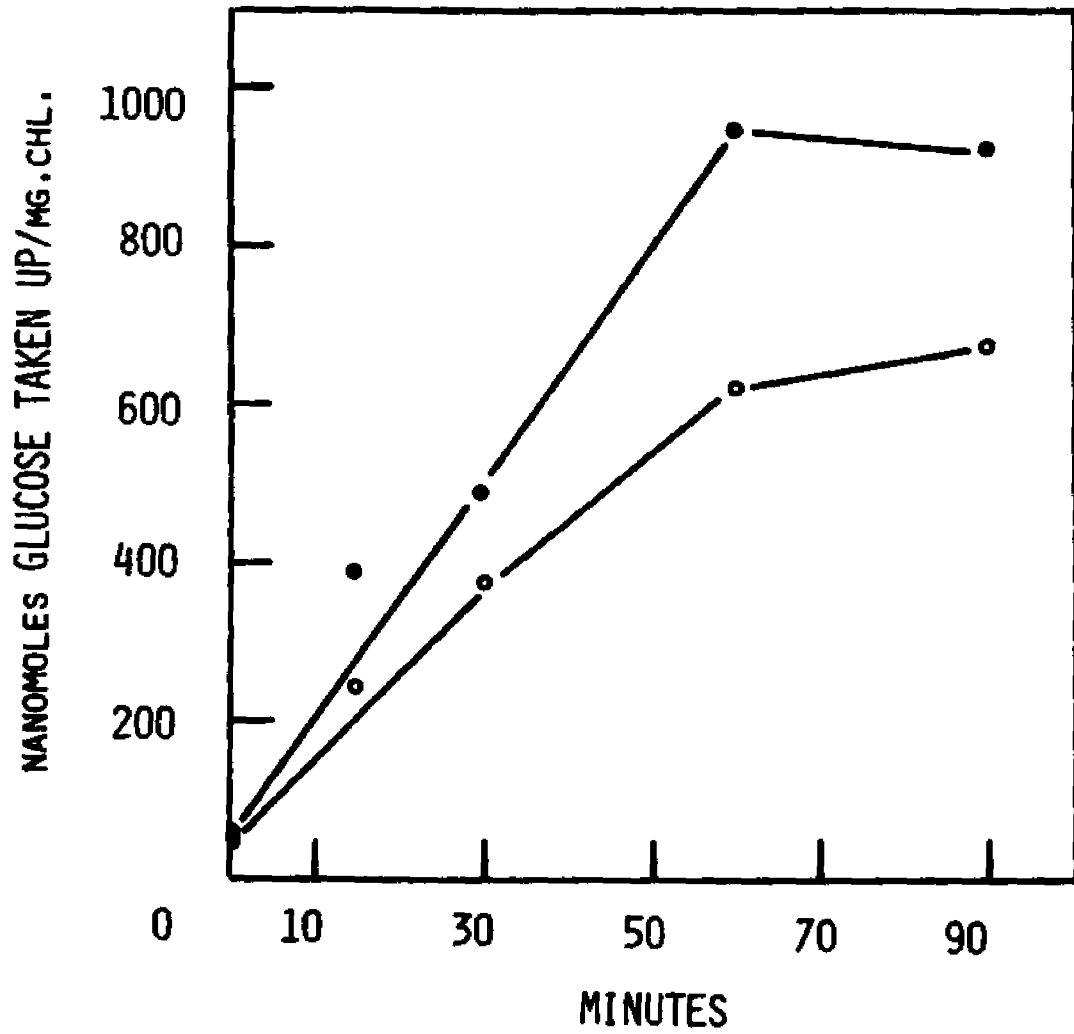
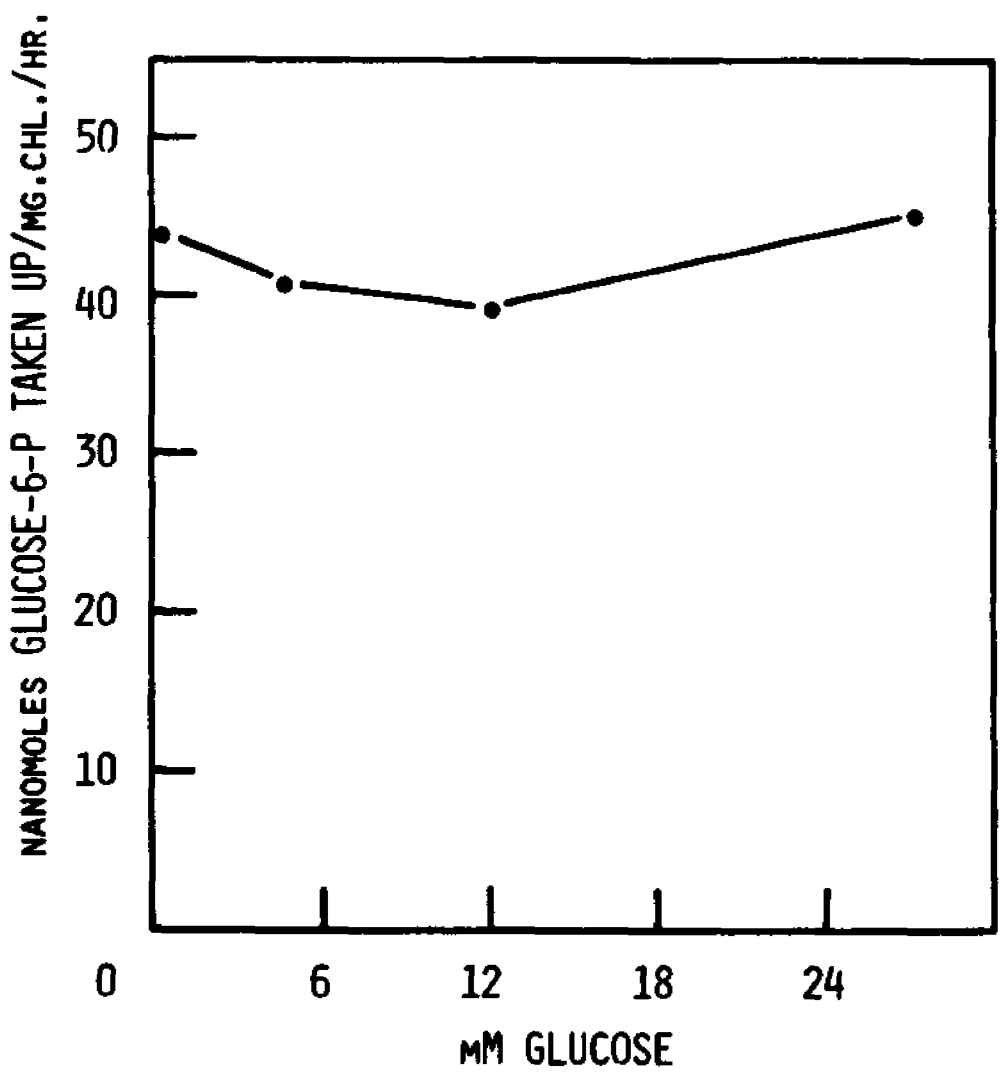

NANOMOLES GLUCOSE TAKEN UP/MG.CHL.
1000
800
600
400
200
0
10
30
50
70
90
MINUTES

Figure 7. The effect of ^{12}C-glucose on the rate of photo-mediated uptake of ^{14}C-glucose 6-P by Anabaena flos-aquae. The assimilation of glucose 6-P was measured as described in the Materials and Methods section D. The 2 ml incubation mixture contained cells equivalent to 250 μg of chlorophyll, 6.3 μmoles of glucose 6-P (specific radioactivity of 0.16 μCi per μmole), and varying amounts of ^{12}C-glucose.


NANOMOLES GLUCOSE-6-P TAKEN UP/MG.CHL./HR.
50
40
30
20
10
0
6
12
18
24
mM GLUCOSE

and Pi to the outside, the ^{12}C-glucose would dilute the released radioactivity and an apparent inhibition of glucose 6-P accumulation would be observed. The reverse experiment was also performed, in which the assimilation of ^{14}C-glucose by Anabaena 1444 was challenged by ^{12}C-glucose 6-P (appendix H). No significant inhibition of glucose accumulation was observed over a concentration range of 0-25 mM glucose 6-P, also indicating that uptake of glucose 6-P did not involve a dephosphorylation to the outside of the cell with the release of glucose and Pi. These experiments do not rule out the possibility of dephosphorylation of glucose 6-P as it transverses the cell envelope.

The results of a glucose 6-P uptake experiment with ^{14}C-(UL)-glucose-6-P , and ^{32}P-glucose 6-P are given in Figure 8. To show that a particular compound, such as a sugar phosphate, is entering the cells intact, it is necessary to demonstrate in a double-label experiment that the sugar moity, glucose, is entering the cells in a similar fashion to the phosphate group.

The treated cells were recovered from this experiment, washed, lysed, and the extracts chromatographed in a descending system with Wood's GW-3 sovent (1961). The chromatograms were then exposed to No-screen X-ray film in order to identify the compounds in which radioisotope was located. The major portion of the radioactivity co-chromatographed with glucose 6-P (Figure 9). The other radioactive

Figure 8. The assimilation of ^{14}C-glucose 6-P and ^{32}P-glucose 6-P by illuminated cells of *Anabaena flos-aquae*. Uptake of glucose 6-P was performed as described in the Materials and Methods section D. The 1 ml incubation mixtures contained cells equivalent to 200 μg chlorophyll, and 5 μmoles glucose 6-P either as ^{14}C-glucose 6-P (specific radioactivity 0.1 μCi/μmole) (○); or as glucose 6-^{32}P (specific radioactivity 0.15 μCi/μmole (●).

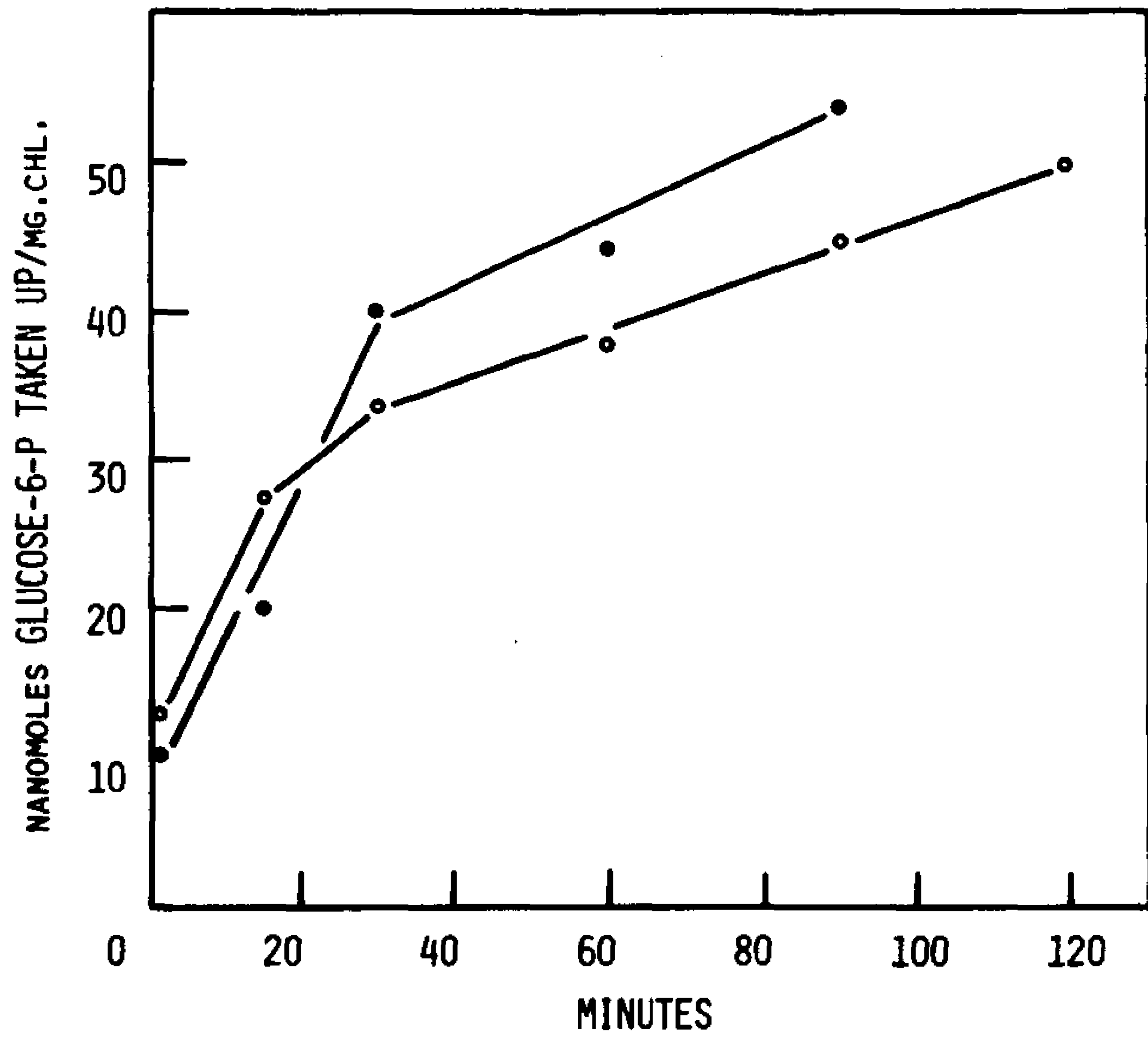
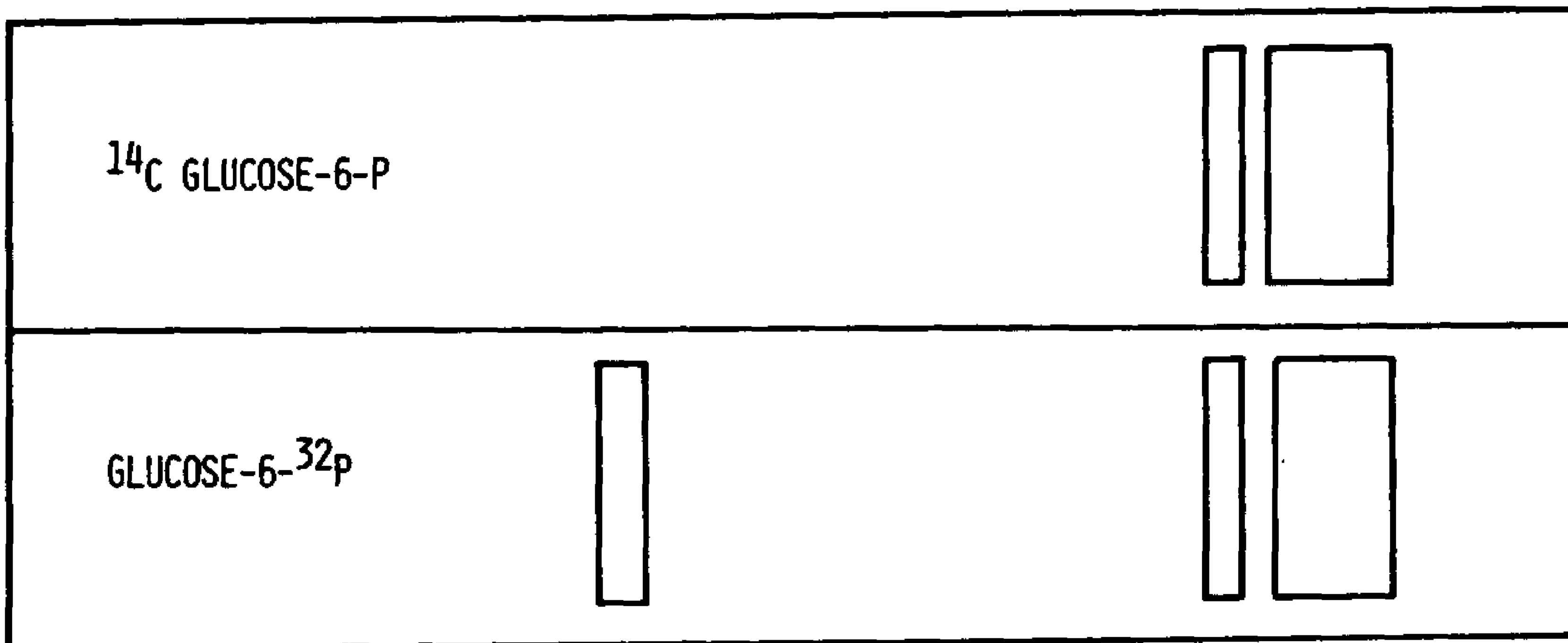

NANOMOLES GLUCOSE-6-P TAKEN UP/MG.CHL.
10
20
30
40
50
0
20
40
60
80
100
120
MINUTES

Figure 9. Chromatographic separation of the assimilated ^{14}C-glucose 6-P and ^{32}P-glucose 6-P. The extraction and chromatographic analysis of the assimilated products were performed as described in the Materials and Methods section F.


^{14}C GLUCOSE-6-P
GLUCOSE-6-^{32}P
ORIGIN

spots were at the R_f values of fructose-6-P or 6-phosphogluconate, and Pi. The percentage distribution of the radioactivity in these recovery experiments were as follows: 1. For ^{14}C-glucose-6-P, 85% of the label was associated with glucose 6-P, and 15% of the label was found either as fructose-6-P or 6-phosphogluconate. 2. For ^{32}P-glucose 6-P, 57% of the label was associated with glucose 6-P, 20% of the label was associated with either fructose-6-P or 6-phosphogluconate, and approximately 23% of the label separated as Pi. In another experiment, the amount of exogenous acid and basic phosphatase activity was determinted to be negligible, with the radioactivity recovered from the medium almost exclusively associated with glucose 6-P. Uptake experiments were also performed with ^{14}C-glucose, ^{14}C-glucose 1-P, and 2-deoxy glucose 6-^{32}P. The chromatographic separation and identification of the assimilated products is shown in appendix I. As shown in this appendix, glucose migrates in a completely different manner than either glucose-6-P or glucose-1-P. In addition, glucose appeared to be taken up in the same manner in the light and dark since the migration patterns were the same from uptake experiments performed under either light condition. It seems therefore that the major amount of glucose 6-P which was assimilated by <u>Anabaena</u> 1444 entered the cells as intact molecules.

D. The Photomediated Assimilation of Glucose 6-P by *Anabaena* 1444.

There are three modes by which microorganisms assimilate nutrients from the external environment (Kornberg, 1973). One is facilitated transport or diffusion, which is the simplest mode for a substance to enter a microbial cell. The substance combines with a protein carrier on one side of the membrane and is released on the other side. Energy is not required for this type of transport system, since movement of the substrate from the medium to the inside of the cell is insured by various intracellular reactions which keep the internal concentrations of the substrate low. In this type of transport system the substance initially appears inside the cell in the same chemical form as it is in the medium.

A second type of uptake system, the phosphotransferase system, was first described by Kundig *et al*. (1964). This system catalyzes the first step in the utilization of some sugars by many types of bacteria. It couples the uptake of the sugars to their phosphorylation and, thus, the sugar taken up appears initially not in the same form in which it was present outside, but as the sugar phosphate.

The most prevalent means for nutrients, including amino acids and many carbohydrates, to enter microorganisms is by an active transport system involving membrane proteins. In active transport the substrate enters the cell in a

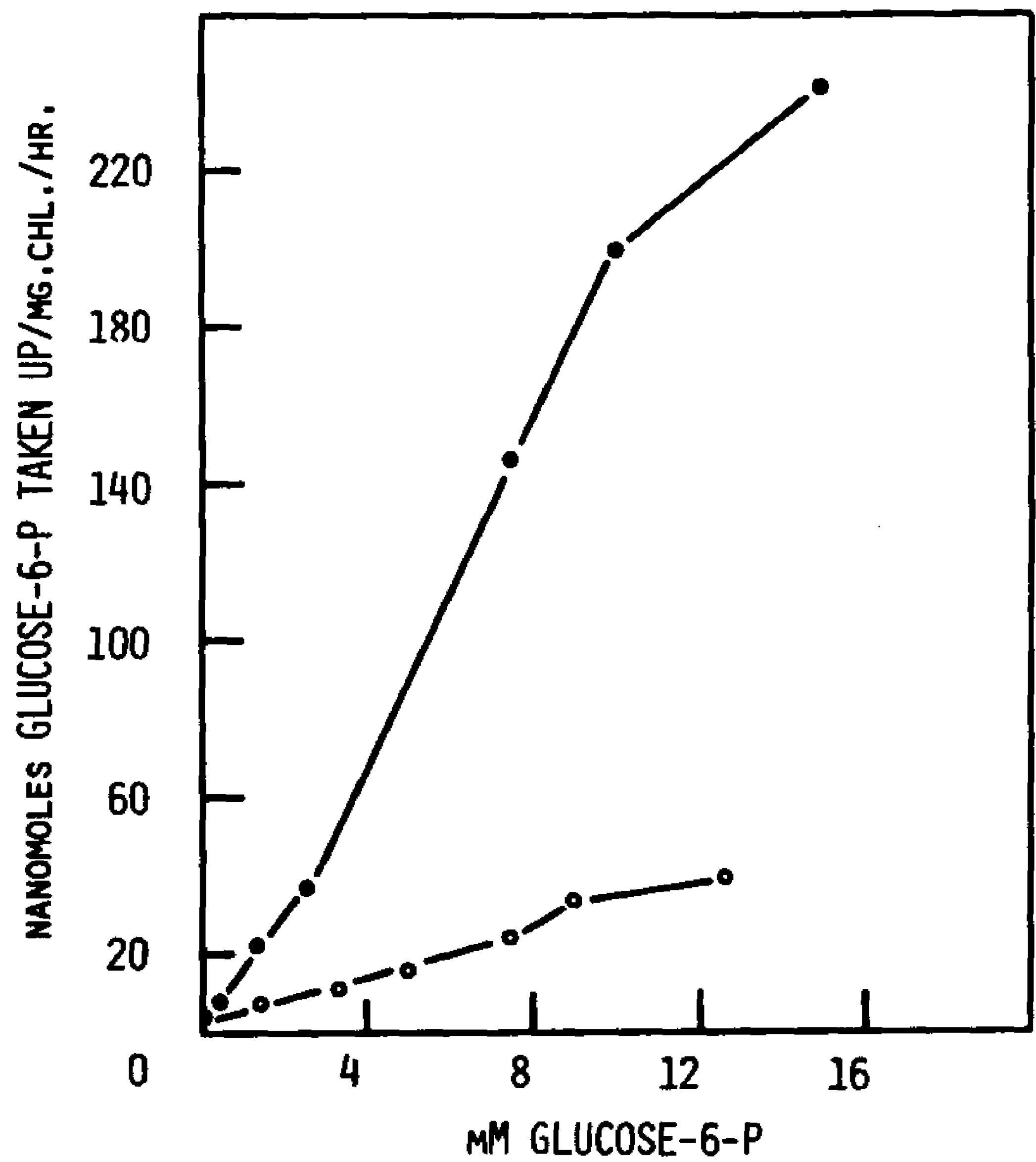
chemically unchanged form. The major difference between this system and facilitated diffusion is the linkage of the transport of the substrate into the cell with the expenditure of energy.

It appears that the uptake of glucose-6-P by *Anabaena* 1444 is performed by an active transport mechanism. One of the characteristics of an active transport mechanism is the similarity between the kinetics of transport and enzymatic reactions; that is, a Michaelis-Menten type analysis can usually be applied to the transport process. This suggests that there are a limited number of specific sites to which the substrate must bind in order to be assimilated by a cell. In Figure 10 saturation curves for photomediated uptake of glucose-6-P and dark assimilation of glucose-6-P are shown. The amount of glucose-6-P taken up in the light is much greater than that taken up in the dark. However, the dark transport of glucose 6-P appeared to saturate at 10 mM glucose 6-P and the light transport of glucose 6-P appeared to saturate at 12 mM. Unlike other active transport systems which have previously been investigated (Winkler, 1973; Tarant and Coleman, 1972) the saturation curves for glucose-6-P influx are not typically hyperbolic, but are more sigmoidal in nature and therefore do not lend themselves to Lineweaver-Burke analysis for a K_M of transport into the cells. By approximating from the sigmoidal saturation curves, only a rough

Figure 10. Saturation curves for glucose 6-P assimilation in *Anabaena flos-aquae* in the light and dark. The uptake of glucose 6-P was followed as described in section D of the Materials and Methods. The incubation mixtures contained cells equivalent to 250 μg chlorophyll, and varying amounts of glucose 6-P (specific radioactivity 0.16 μCi/μmole).

Assimilation in the light (●)

Assimilation in the dark (○)


NANOMOLES GLUCOSE-6-P TAKEN UP/MG.CHL./HR.
220
180
140
100
60
20
0
4
8
12
16
mM GLUCOSE-6-P

estimation of the K_M ($S_{0.5}$) can be made. For the photo-stimulated uptake the $S_{0.5}$ for glucose 6-P is 6.3 mM and for the dark mediated uptake it is 5.8 mM.

The similarities between the $S_{0.5}$ for uptake in the light and dark are interesting for the following reasons. The increased uptake in the light suggests an active transport system; if this is the case, the energy is not utilized to lower the K_M of the transport system for glucose-6-P. In *E. coli* the transport system for glucose-6-P utilizes the energy which is required for assimilation to occur to lower the K_M for glucose 6-P (Winkler, 1973). On the other hand, the transport systems for the βgalactosides and amino acids are also energy dependent, but unlike the *E. coli* glucose-6-P system, the energy is not utilized to increase the affinity of the uptake system for the substrate (lower the K_M), but is spent to raise the K_M for efflux (Winkler and Wilson, 1966). The assimilation of βgalactosides in *E. coli* occurs by facilitated diffusion and once the molecule enters the cell, the lowered affinity of the carrier protein for exit of the galactosides tends to keep the substance inside the cell. Thus it is not unusual to find an active transport system where the energy is not expended on decreasing the carriers' K_M for the substrate.

The other possibility is that the mechanism of uptake is the same in the light and dark, but the increased accumulation in the light is due to the increased

energy produced during photophosphorylation (see later discussions).

The implication throughout this discussion is that the glucose-6-P uptake system is a carrier-mediated system. In certain gram-negative bacteria, external proteins, some of which are believed to be specific permeases or binding proteins, are released into the medium following a gentle osmotic shock treatment (Heppel, 1969). The blue-green algae have a cell wall which is very similar to the Gram-negative bacteria, and it was hoped that by utilizing an osmotic shock technique a specific permease for glucose-6-P could be released from the cells. Heppel's (1969) method of osmotic shock was followed: 1) cells are suspended in 80 parts of 0.5 M sucrose in 33 mM Tris-HCl, pH 7.2, and 1×10^{-4} M EDTA; 2) cells are quickly collected and resuspended in 80 parts of cold 5×10^{-4} M $MgCl_2$. 3) The cells are then collected and the supernatant from the last step is the shock fluid containing the released permeases. Following this method no detectable loss in the alga's ability to assimilate glucose 6-P from the medium was detected. This could be due to some inherent difference in the cellular morphology between the blue-green algae and the Gram-negative bacterium _E. coli_. Lang (1968) described a very intimate and close relationship between the cell wall (L1 layer) and the plasmalemma, and suggested that there was no periplasmic space in the blue-green algae equivalent to

that of the _E. coli_ strain which was postulated to contain the permeases. This may be one of the many possible reasons why there was no detectable release of a carrier protein for glucose-6-P uptake. Heppel's method therefore would need some modifications to be effective in these algae, since it was known to be of only limited effectiveness when used for Gram-negative bacteria other than _E. coli_.

If glucose 6-P is assimilated in _Anabaena_ 1444 by means of a carrier mediated system it might be possible to inhibit the uptake of glucose-6-P by challenging the assimilatory system with various sugar phosphates. In _E. coli_ Pogell _et al_. (1966) found that, in cells induced for the uptake of glucose 6-P, various sugar phosphates were able to inhibit the accumulation of glucose-6-P. They found that glucose-1-P, and fructose-1-P inhibited the uptake of glucose-6-P by at least 52%, but that ribose-5-P, fructose 1,6-diP, and glucose-6-S were not very effective inhibitors of the assimilation of glucose-6-P.

The photomediated uptake of glucose-6-P in _Anabaena_ 1444 was challenged by various sugar phosphates (Table III) under conditions of limiting glucose-6-P (3.8 mM) so that any competitive inhibition which the various phosphorylated compounds might exhibit could be detected. As discussed previously, glucose had no effect on the uptake of glucose 6-P. The two most effective competitive inhibitors were glucose-1-P and fructose-6-P, which had a maximal inhibition

TABLE III. THE INHIBITION OF GLUCOSE-6-PHOSPHATE UPTAKE BY VARIOUS PHOSPHORYLATED METABOLITES.

THE EFFECT OF VARIOUS SUGAR PHOSPHATES ON GLUCOSE-6-P UPTAKE IN ANABAENA. THE EXPERIMENTS WERE PERFORMED IN THE LIGHT IN A VOLUME OF 2 ML IN WHICH THERE WERE CELLS EQUIVALENT TO 250 μG CHLOROPHYLL, AND 3.8 μMOLES OF G6P CONTAINING 1 μCI OF (UL) ^{14}C-GLUCOSE-6-P. SAMPLES OF 100 μL WERE WITHDRAWN, WASHED TWICE IN 6 ML OF BRISTOL'S SOLUTION AT PH 8.2, RESUSPENDED IN 3 ML OF THE SAME, FILTERED ONTO WHATMAN GLASS FIBER FILTERS AND RADIOACTIVITY WAS DETERMINED WITH A PLANCHET COUNTER. THE CONTROL RATE OF GLUCOSE-6-P ENTRY INTO THE CELLS WAS 35 NMOLES/MG CHL · HR.

METABOLITE	CONCENTRATION (MM)	% INHIBITION
GLUCOSE-6-PHOSPHATE	3.3	50*
GLUCOSE-1-PHOSPHATE	8.8	54
FRUCTOSE-6-PHOSPHATE	6.5	42
FRUCTOSE 1,6 DIPHOSPHATE	0-8.8	NONE
6-PHOSPHOGLUCONATE	0-8.8	NONE
RIBOSE-5-PHOSPHATE	0-7	NONE
GLUCOSE	0-27	NONE

* ISOTOPE DILUTION

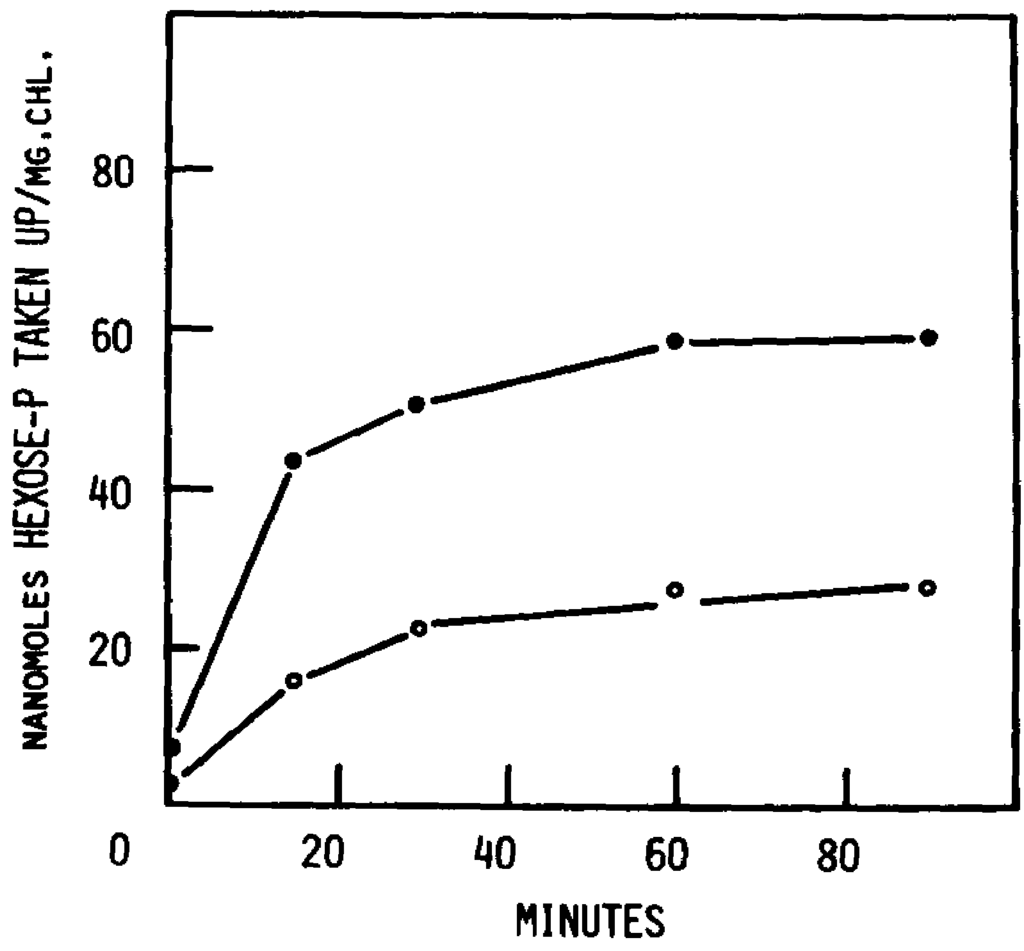
of glucose-6-P uptake of 54% and 42% respectively as compared to the control level of incorporation. Fructose 1,6-diP, 6-phosphogluconate, and ribose-5-P were without effect on the assimilation of glucose-6-P by Anabaena 1444. These findings are qualitatively similar to those of Pogell (1966) with E. coli. In Anabaena the maximum inhibition caused by the competitive sugar phosphate was much less than that which was observed in the E. coli system. The relative ineffectiveness of the competing sugar phosphates in inhibiting the assimilation of glucose-6-P in Anabaena 1444 might indicate that its sugar phosphate uptake system is more specific for glucose-6-P accumulation than the E. coli system. In Figure 11, a comparison between the rates of light mediated glucose-6-P and glucose-1-P uptake are shown. As seen from these results glucose-1-P was accumulated by the cells at about 50% the rate at which glucose-6-P was accumulated. This supports the finding that glucose-1-P was only a moderately effective competitive inhibitor of glucose-6-P assimilation. It thus appears that glucose-6-P is the best substrate for the uptake system, being transported to a greater extent than the other sugar phosphates.

The assimilation of glucose-6-P by Anabaena 1444 in the light as compared to the dark was previously shown to be much greater. This suggested the possibility that the uptake of glucose-6-P was energy dependent, since the

Figure 11. The comparison of photomediated glucose 6-P and glucose 1-P uptake by _Anabaena_ _flos-aquae_. The assimilation of the sugar phosphates was followed as described in section D of the Materials and Methods. The assays contained 250 μg chlorophyll and either 6.3 μmoles of glucose 6-P (specific radioactivity of 0.16 μCi/μmole) or 6.3 μmole of glucose 1-P (specific radioactivity of 0.8 μCi/ μ mole).

Glucose 6-P (●)

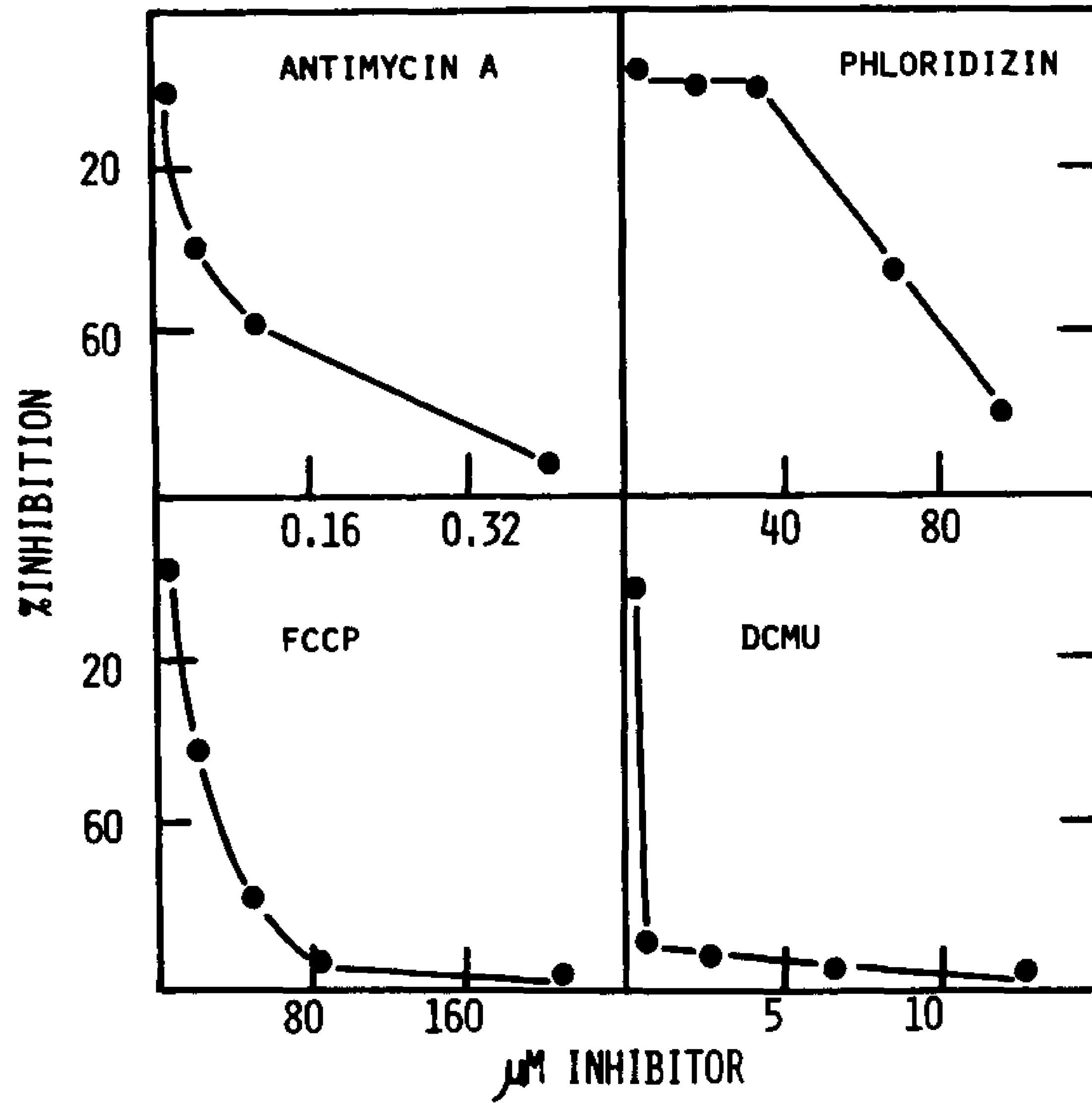
Glucose 1-P (○)


NANOMOLES HEXOSE-P TAKEN UP/MG.CHL.
80
60
40
20
0
20
40
60
80
MINUTES

blue-green algae produce much greater amounts of energy during photosynthesis than during aerobic respiration (Leach and Carr, 1970). The enhanced transport of glucose 6-P in the light was known to be a process independent of its turnover by way of the pentose phosphate pathway. The inhibition of glucose-6-P oxidation in the light was shown by Pelroy and Bassham (1972), and as previously discussed has been attributed to the inhibition of the enzyme glucose 6-P dehydrogenase, by either RuDP or ATP, and NADPH (Grossman and McGowan, 1975; Pelroy _et al_., 1972). The enhanced uptake of glucose-6-P in the light was therefore not linked to increased utilization of glucose-6-P since the pentose phosphate pathway is maximally operative in the dark when glucose 6-P assimilation was minimal. The enhanced uptake of glucose-6-P in the light was therefore thought to be linked to some aspect of photophosphorylation.

The existence of a light dependent cyclic electron transport system coupled to ATP production or a high energy intermediate was demonstrated in the blue-green algae by Teichler-Zallen (1967). Using the photosynthetic inhibitors DCMU, Antimycin A, and FCCP, the possibility was investigated that the photoassimilation of glucose-6-P was coupled to the energy derived during the cyclic electron flow. In Figure 12 are presented the effects of various photosynthetic inhibitors, including phloridizin,

Figure 12. The effect of various photosynthetic inhibitors on CO_2 fixation in the blue-green alga *Anabaena flos-aquae*. Experiments were performed as described in the Materials and Methods. The radioactive assay contained 250 μg chlorophyll, 25 μmoles HEPES at pH 8.0, and 12.5 μmoles of HCO_3^- (specific radioactivity 0.66 μCi/μmole).



ANTIMYCIN A
PHLORIDIZIN
FCCP
DCMU
20
60
20
60
0.16
0.32
40
80
80
160
5
10
%INHIBITION
μM INHIBITOR

on the rate of CO_2 fixation, a parameter also requiring ATP production.

Antimycin A was shown by Tagaua *et al*. (1963) to be an inhibitor of ferredoxin-catalyzed cyclic photophosphorylation in chloroplasts at concentrations at which non-cyclic photophosphorylation remains unaffected. This finding was later extended to whole cells where it was shown that Antimycin A selectively inhibited processes requiring only ATP, that is processes that could be supported solely by cyclic photophosphorylation (Tanner *et al*., 1965; Schurmann *et al*., 1971). In *Anabaena* 1444, Antimycin A was found to be a potent inhibitor of CO_2 fixation; at a concentration of 0.36 μM Antimycin A, CO_2 fixation was inhibited 100%.

The inhibition of CO_2 fixation caused by DCMU is also shown in this figure; DCMU at a concentration of 10 μM inhibited CO_2 fixation 100%. Avron and Shavit (1965) have shown that DCMU inhibits photosynthesis by preventing the photoreduction of $NADP^+$. This inhibition can be overcome by the addition of ascorbate-DCIP, indicating an inhibition of non-cyclic electron flow.

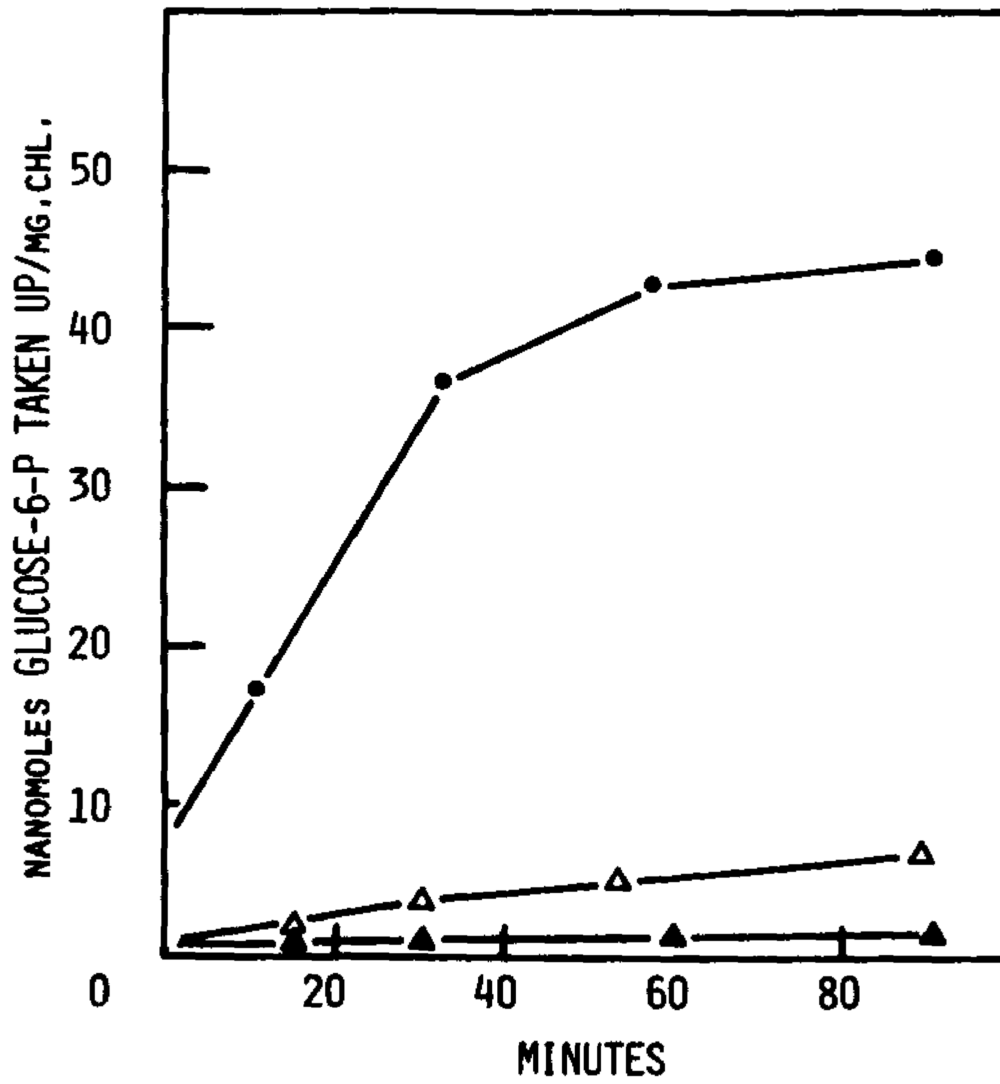
FCCP was shown by Avron and Shavit (1965) to be a very potent uncoupler of photophosphorylation. In *Anabaena* 1444 FCCP inhibited CO_2 fixation fully at a concentration of 100 μM. A comparison of the effects of these various inhibitors on the rate of photostimulated

glucose-6-P assimilation and on CO_2 fixation was performed. In Figure 13 the effects of FCCP on the rate of glucose-6-P uptake in the light are given. At a concentration of 100 μM FCCP, the rate of glucose-6-P accumulation was inhibited by at least 85% as compared to the control rate. This concentration of FCCP, as mentioned above, gave 100% inhibition of CO_2 fixation. It therefore appeared that glucose-6-P uptake in the light was coupled to the energy released during photosynthetic electron flow, and possibly to the photophosphorylation occurring during this electron flow.

In Figure 14 are shown the effects of DCMU, an inhibitor of non-cyclic electron transport, on the rate of photomediated glucose 6-P uptake. DCMU at a concentration of 15 μM, which was 1.5 times the concentration needed to give 100% inhibition of CO_2 fixation in this alga, only gave a 50% inhibition of glucose-6-P uptake. Using a higher concentration, 50 μM DCMU, (which is five times the necessary concentration to completely inhibit CO_2 fixation), glucose 6-P uptake in the light was still not completely inhibited. These data indicate that if glucose 6-P uptake was dependent on energy derived from photophosphorylation it was not closely dependent on energy derived from non-cyclic photophosphorylation, but on energy from cyclic photophosphorylation.

Figure 13. The effect of the uncoupler FCCP on the photo-mediated uptake of glucose 6-P by *Anabaena flos-aquae*. The assimilation of glucose 6-P was followed as described in the Materials and Methods section D. Each assay contained cells equivalent to 250 μg chlorophyll, and 6.3 μmoles glucose 6-P (specific radioactivity 0.16 μCi/μmole).

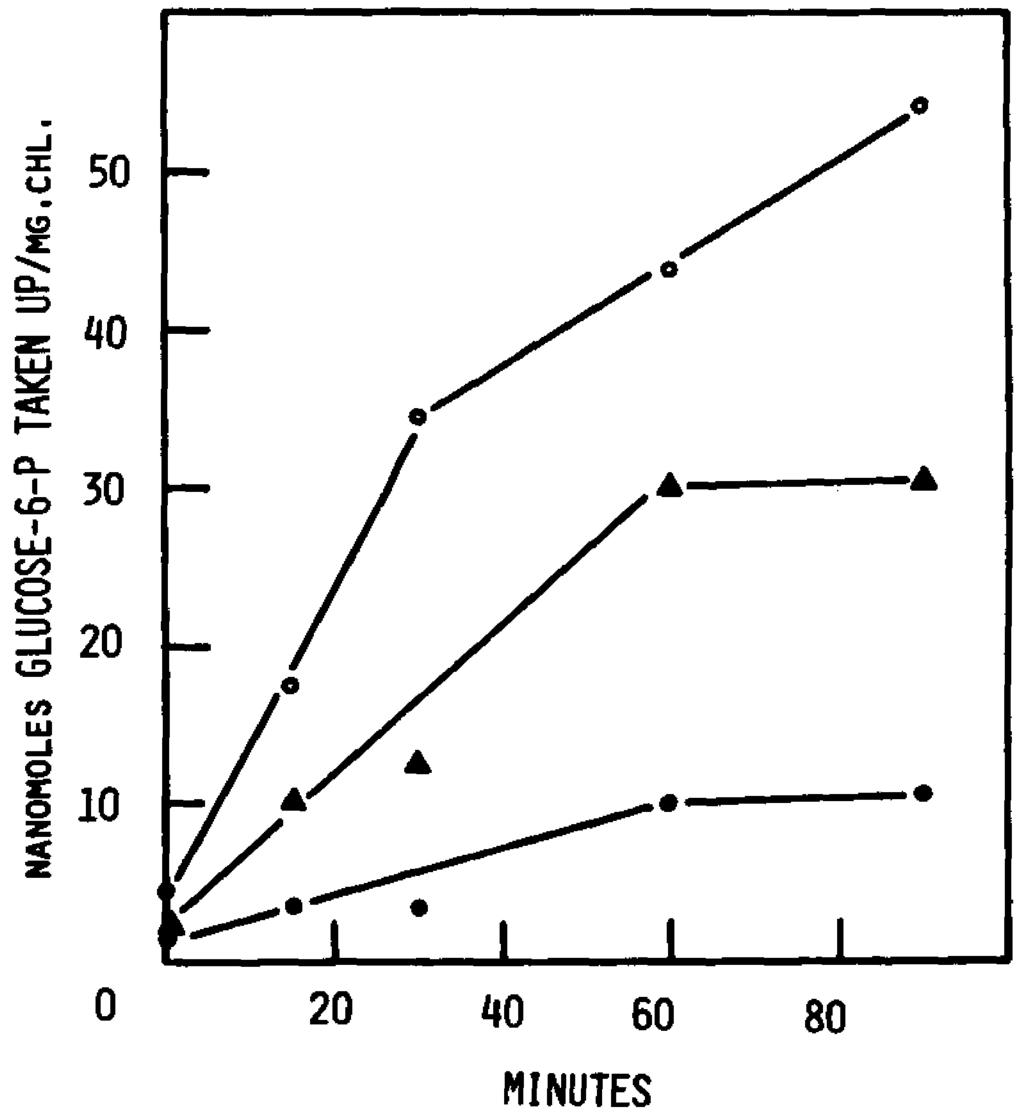
Control	(●)
100 μM FCCP	(△)
150 μM FCCP	(▲)



NANOMOLES GLUCOSE-6-P TAKEN UP/MG.CHL.
50
40
30
20
10
0
20
40
60
80
MINUTES

Figure 14. The effect of DCMU on the photomediated assimilation of glucose 6-P by *Anabaena flos-aquae*. The experiments were performed as previously described in the Materials and Methods section D. The radioactive assays contained cells equivalent to 250 μg chlorophyll, and 6.3 μmoles of glucose 6-P (specific radioactivity 0.16 μCi/μmole).

Control	(○)
15 μM DCMU	(▲)
50 μM DCMU	(●)


NANOMOLES GLUCOSE-6-P TAKEN UP/MG.CHL.
0
10
20
30
40
50
20
40
60
80
MINUTES

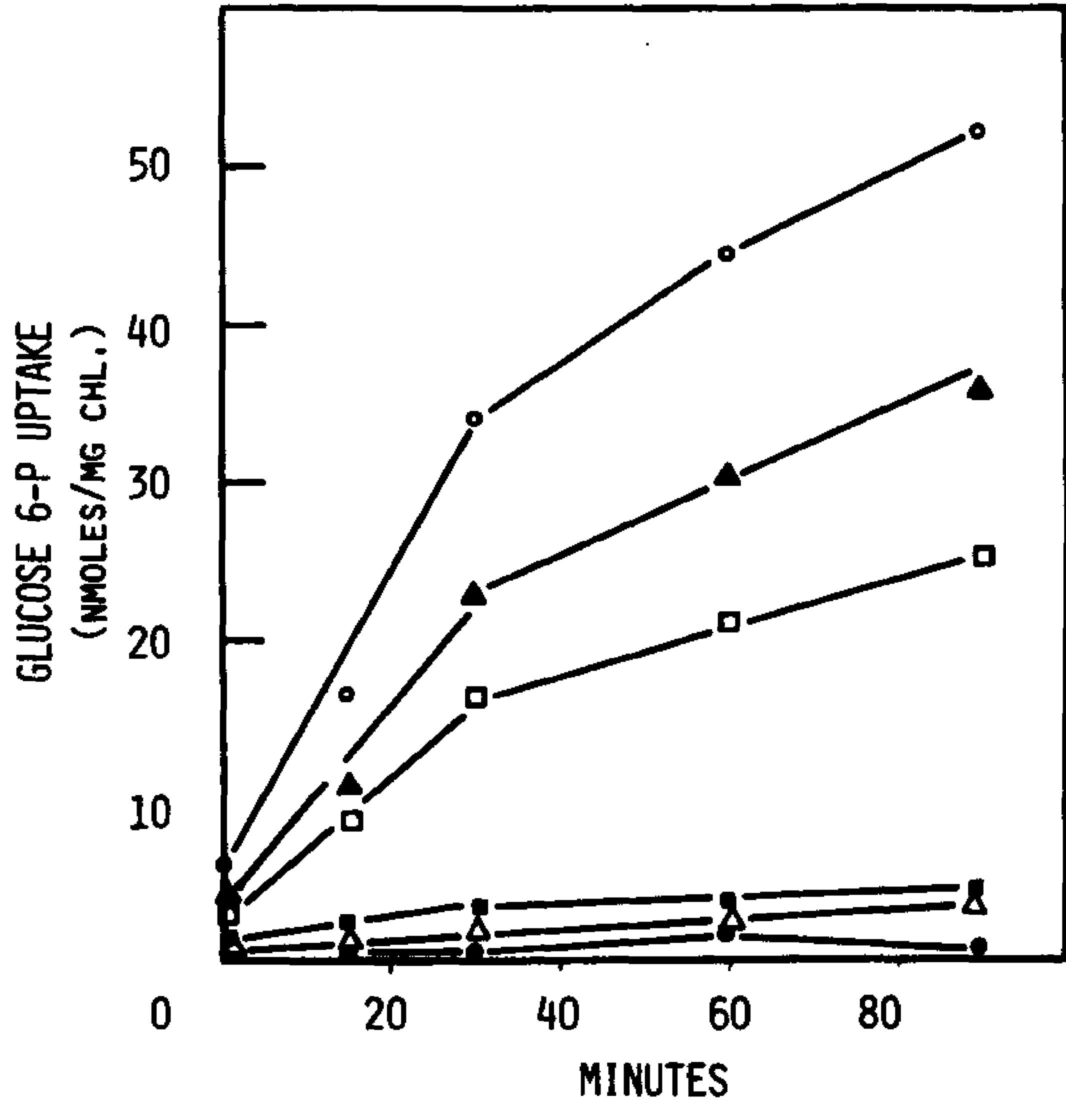
The effects of the inhibitor Antimycin A on the photomediated uptake of glucose-6-P are shown in Figure 15. Antimycin A is a specific inhibitor of ferredoxin-catalyzed cyclic photophosphorylation. As shown in this figure glucose-6-P uptake in the light was much more sensitive to this inhibitor than to DCMU. At a concentration of 0.25 μM, Antimycin A caused a 50% inhibition of glucose-6-P uptake compared to a 65% inhibition of CO_2 fixation. These results demonstrated a closer dependence of glucose 6-P uptake on cyclic phosphorylation than on non-cyclic electron flow. Also shown in this figure is the finding that 150 μM phloridizin completely inhibited the uptake of glucose-6-P in the light. Phloridizin is a specific energy transfer inhibitor, which is believed to act by inhibiting the Ca^{+2} and Mg^{+2} dependent ATPases (Izawa and Good, 1972). The assimilation of glucose-6-P by Anabaena 1444 therefore appeared to be energy dependent, requiring ATP which was predominately formed during cyclic electron flow.

E. The Oxidative Respiration of Exogenously Supplied Glucose-6-P.

The uptake of glucose-6-P in the light has been demonstrated to be energy dependent, and independent of the rate of utilization. The pentose phosphate pathway has been shown to be the major dissimilatory pathway for glucose-6-P. Pelroy and Bassham (1972) demonstrated that

Figure 15. The effects of Antimycin A and phloridizin on the rate of photomediated uptake of glucose 6-P by *Anabaena flos-aquae*. The experiments were performed as described in section D of the Materials and Methods. The assays contained 250 μg of chlorophyll, and 6.3 μmoles of glucose 6-P (specific radioactivity of 0.16 μCi/μmole).

Control	(○)
0.10 μM Antimycin A	(▲)
0.25 μM Antimycin A	(□)
2.5 μM Antimycin A	(■)
150 μM Phloridizin	(△)
Heat killed cells	(●)


GLUCOSE 6-P UPTAKE
(NMOLES/MG CHL.)
0
10
20
30
40
50
20
40
60
80
MINUTES

the internal pool of glucose-6-P did not turn over in the light, but did in the dark. This photoinhibition of glucose 6-P oxidation, by way of the pentose phosphate pathway, was linked to the inhibition of the first enzyme of the pathway, glucose 6-P dehydrogenase (Pelroy *et al*., 1972). As previously mentioned, RuDP, ATP, and NADPH were suggested as possible effectors of this inhibition. To investigate the metabolism of exogenously supplied hexose-6-P, the oxidation of exogenously supplied ^{14}C-1-glucose 6-P and ^{14}C-1-glucose was followed both in the light and dark. The oxidation of the glucose and glucose-6-P was monitored by measuring the C-1 which was respired as CO_2 during the oxidation of 6-phosphogluconate in the pentose phosphate pathway.

As shown in Table IV, there was an insignificant amount of CO_2 respired from either glucose or glucose 6-P in the light indicating that the pentose phosphate pathway was inoperative. When the cells were switched to the dark the amount of CO_2 respired from both glucose and glucose-6-P was greatly (over ten fold) increased. The experiments were performed in a closed system in which the respired CO_2 was trapped in a KOH center well. It was therefore possible that the decrease in respired CO_2 in the light was due to photosynthetic refixation of the CO_2 released. Although this possibility cannot be ignored in a closed system, evidence presented by other workers and discussed

TABLE IV. THE TURNOVER OF EXOGENOUSLY SUPPLIED ^{14}C-1 GLUCOSE AND ^{14}C GLUCOSE-6-P BY *ANABAENA FLOS-AQUAE*.

THE 1 ML ASSAYS CONTAINING 25 μMOLES OF RADIOACTIVE METABOLITE WERE PERFORMED IN THE CLOSED SYSTEM DESCRIBED IN MATERIALS AND METHODS SECTION E.

CONDITIONS	ATMOSPHERE	INHIBITOR	CO_2 RELEASED FROM GLUCOSE
			NANOMOLES/MG PROTEIN · 5 HR
LIGHT	AIR	--	4
DARK			64
LIGHT	AIR	125 μM DCMU	51
DARK			56
LIGHT	AIR	100 μM FCCP	50
DARK			46
LIGHT	AIR	10 μM ANTIMYCIN A	64
DARK			68

TABLE IV. (CONTINUED)

CONDITIONS	ATMOSPHERE	INHIBITOR	CO_2 RELEASED FROM GLUCOSE-6-P
			NANOMOLES/MG PROTEIN · 5 HR
LIGHT	AIR	--	6.4
DARK			68
LIGHT	N_2/CO_2 FREE AIR	-	5.5
DARK			62
LIGHT	AIR	125 μM DCMU	46
DARK			68
LIGHT	AIR	100 μM FCCP	73.8
DARK			62

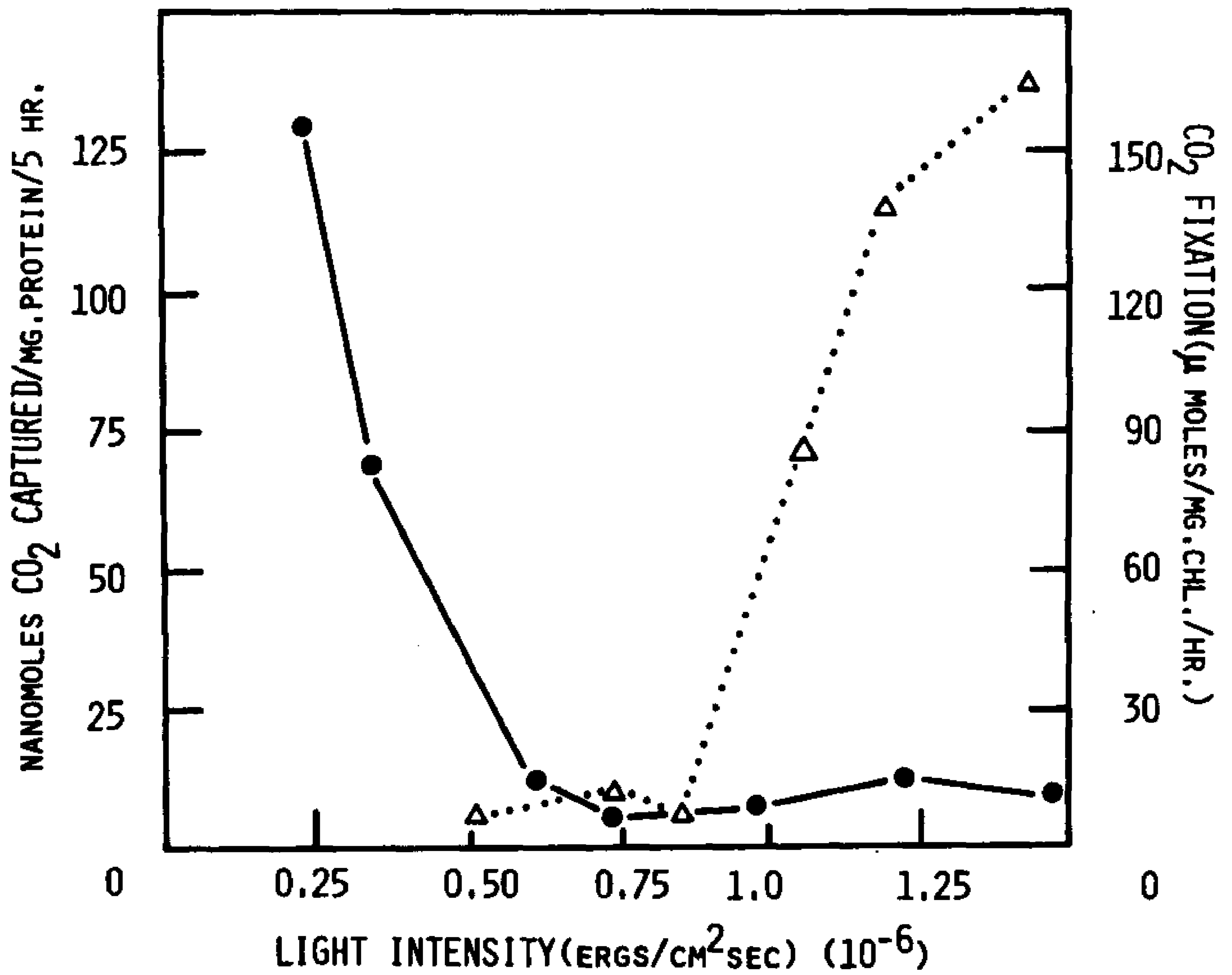
previously suggested that CO_2 is not respired in the light in our system. To further confirm that the lack of CO_2 captured in the light was not the result of a photosynthetic refixation, the effect of light intensity on CO_2 release from glucose 6-P and on CO_2 fixation was examined. In Figure 16, light intensity is plotted versus µmoles of CO_2 fixed and nmoles of CO_2 captured. As shown in this figure, CO_2 fixation decreased with decreasing light intensity and oxidation of glucose 6-P by way of the pentose phosphate pathway increased with decreasing light intensity, thus the two processes were inversely related. The interesting part of this curve however, was the area where the two processes overlap (between the light intensities of 0.5-0.9 x 10^6 ergs/cm^2·sec). Although the rate of CO_2 fixation was 1000 fold greater than the rate of glucose 6-P incorporation, there was no correlation between CO_2 release and the rate of fixation in this light intensity range. These results suggest but do not prove that the limited CO_2 captured in the light was not due to a photosynthetic refixation, but to an inhibition of the pentose phosphate pathway.

In the dark there were 68 nmoles of CO_2 released from glucose 6-P/mg protein·5 hr, indicating that only 0.33-0.5 of the glucose-6-P taken up by the cells during this period is oxidized by the pathway. Various photosynthetic inhibitors were also tested to determine if the

Figure 16. The effect of light intensity on the rate of CO_2 fixation and glucose 6-P oxidation. The CO_2 fixation was performed as described in the Materials and Methods section C, and glucose 6-P oxidation was followed as described in section E of the Materials and Methods.

CO_2 fixation (△)

Glucose 6-P Turnover (●)



NANOMOLES CO_2 CAPTURED/MG.PROTEIN/5 HR.
125
100
75
50
25
0
150
120
90
60
30
0
CO_2 FIXATION(μ MOLES/MG.CHL./HR.)
0.25
0.50
0.75
1.0
1.25
LIGHT INTENSITY(ERGS/CM^{2}SEC) (10^{-6})

rates of CO_2 release from glucose-6-P oxidation in the light could be increased. As shown in Table IV, both DCMU and FCCP increased the oxidation of glucose-6-P in the light. The mechanism of this increase still cannot be critically evaluated. From the previous work of Pelroy, Rippka, and Stanier (1972) and Grossman and McGowan (1975) it was postulated that certain products of photosynthesis feedback and inhibit the enzyme glucose-6-P dehydrogenase, the first enzyme of the pentose pathway. The photomediated inhibition of glucose 6-P dehydrogenase then results in the observed decrease in CO_2 release from glucose-6-P in the light. The inhibitors DCMU and FCCP are believed to increase CO_2 release from glucose-6-P in the light by decreasing the ATP and NADPH production and therefore resulting in relief of the dehydrogenase from feedback inhibition. The possibility that these inhibitors were acting by preventing the refixation of CO_2 in the closed system cannot be ruled out.

The ability of exogenous glucose-6-P to be metabolized in the dark by way of the pentose phosphate pathway may allow for at least a limited amount of active metabolic work in the dark. The studies of Leach and Carr (1970) demonstrated the existence of a coupled NADPH oxidase system in these algae. It should therefore be possible for blue-green algae to utilize exogenously supplied glucose-6-P to support at least limited metabolic work in

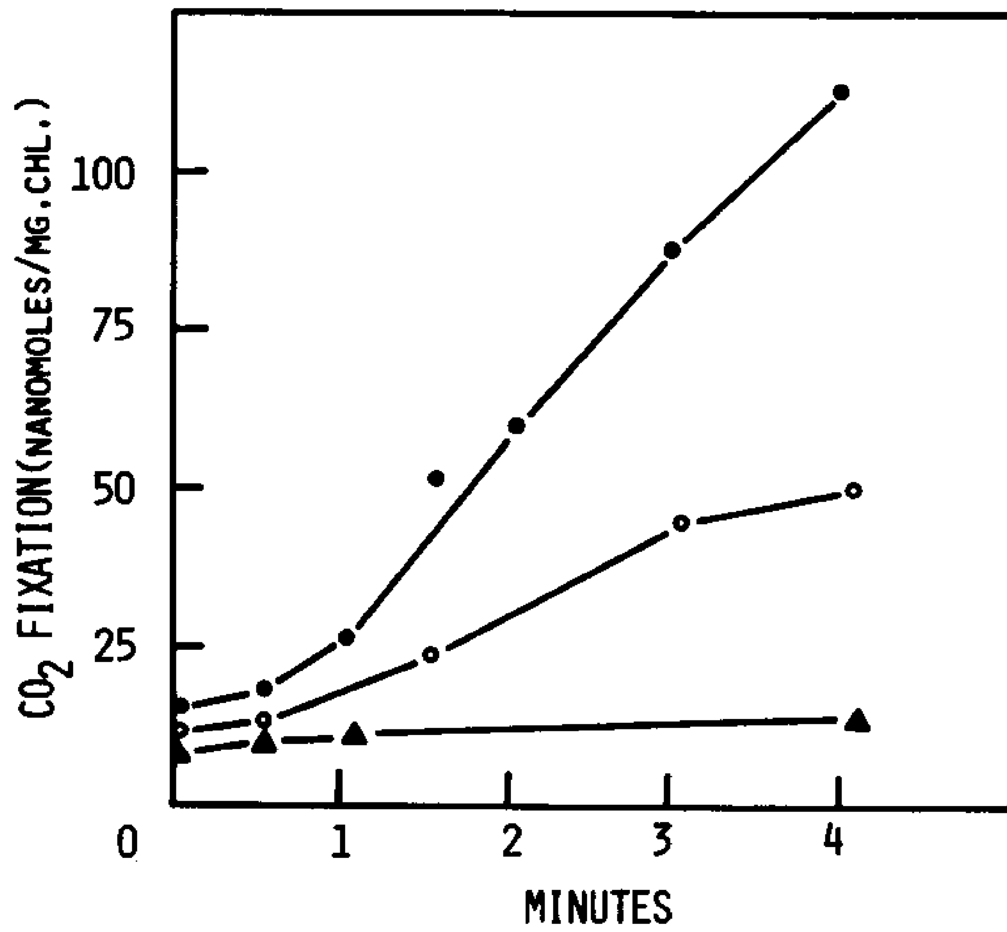
the dark. Padan _et al_. (1970, 1971), studying the reproduction of the cyanophage LPP1G in its host _Plectonema boryanum_, found that the reproductive cycle of the virus could be partially supported by energy produced during aerobic respiration. This was one of the first demonstrations in the blue-green algae of an active metabolic process being supported by a dark oxidase system. In Figure 17 the effects of exogenously supplied glucose-6-P on the rate of dark CO_2 fixation are presented. As previously described, the major autotrophic pathway for CO_2 fixation in the blue-green algae is believed to be the Calvin cycle. This cycle requires both ATP and NADPH to be fully operative. Based on Padan's and others' findings it was thought that exogenously supplied glucose-6-P, which can be oxidized in the dark producing NADPH, and ATP by way of the NADPH oxidase system, should be able to support dark CO_2 fixation. As shown in Figure 17, fixation of CO_2 in the dark was negligible: however, if the cells are exposed to ten minutes of light prior to the start of the dark CO_2 fixation period a large stimulation in the rate of CO_2 fixation was observed. This stimulation can be viewed as being due to a prior buildup of the internal pools of Calvin cycle intermediates, particularly glucose-6-P, 3-phosphoglycerate, and ribulose 1,5-diP. Inhibitors of photosynthesis such as Antimycin A and DCMU, if added to the reaction during the ten minute

Figure 17. The effect of glucose 6-P on the rate of dark CO_2 fixation by Anabaena flos-aquae. Fixation of CO_2 was measured as described in the Materials and Methods, section C. The incubation mixture contained cells equivalent to 250 µg chlorophyll, 12.5 µmoles of $NaH^{14}CO_3$ (specific radioactivity 0.66 µCi/ µmole), and 25 mM HEPES at pH 8.0. The dark controls were maintained in darkness for 2 hr prior to the addition of $NaH^{14}CO_3$ and subsequent to the addition of the $NaH^{14}CO_3$. The preilluminated cells were exposed to 10 minutes of illumination prior to the addition of $NaH^{14}CO_3$ in the dark. The assays containing glucose 6-P were incubated with the sugar phosphate for 2 hr in the dark prior to the addition of the $NaH^{14}CO_3$.

Control - 2 hr dark (▲)

Preilluminated - 10 min. (○)

Glucose 6-P - treated (●)



CO_2 FIXATION(NANOMOLES/MG.CHL.)
100
75
50
25
0
1
2
3
4
MINUTES

illumination, inhibited the subsequent dark CO_2 fixation period. DCMU if added after the preillumination period, but during the dark CO_2 fixation period, had no effect. These results indicated that dark CO_2 fixation was supported by a product, such as glucose-6-P, generated during the active period of photosynthesis.

When the blue-green algal cells were switched to the dark the ribulose 1,5-diP and glucose 6-P pools were quickly depleted and the 3-phosphoglycerate pool showed a rapid increase and then stabilized. The turnover of glucose-6-P in the dark was previously discussed and it is believed that part of the enhanced dark CO_2 fixation was supported by this oxidation. To further confirm this hypothesis _Anabaena_ 1444 was incubated in the dark for 2 hrs. in the presence of glucose-6-P prior to the start of the experiment. As shown in Figure 17, the pretreatment with glucose-6-P enhances the rate of CO_2 fixation in the dark to a much greater extent than the ten minute pre-illumination and indicated that the energy produced during the oxidation of glucose 6-P could be used to support active metabolic work.

The ability of the aerobic dark respiratory pathway to support active metabolic work was not only exemplified in the glucose-6-P enhancement of dark CO_2 fixation, but also in the dark assimilation of glucose-6-P by intact cells of _Anabaena_ 1444. The photomediated assimilation of

glucose-6-P was shown to be an energy dependent process, but the possibility that the dark uptake of glucose-6-P was also energy dependent was not ruled out. In blue-green algae the only feasible source of energy in the dark is the NADPH dependent oxidase system. As discussed above, the major dissimilatory pathway for glucose-6-P is the pentose phosphate pathway; the glycolytic pathway is in-operative so that no substrate level phosphorylation is occurring. Based on the findings of Goldberg and McGowan (1975) (see conclusion) the uptake of glucose 6-P in the dark was investigated using various inhibitors of the dark respiratory pathway of Anabaena 1444. The effects of electron transport inhibitors on the rate of dark glucose-6-P accumulation by intact cells of Anabaena 1444 are shown in Figure 18. Amytal (250 μM), SHAM (2.5 mM), Rotenone (200 μM), and Antimycin A (200 μM) inhibited the dark glucose-6-P accumulation by at least 42%. These results indicated that for dark glucose-6-P transport to occur there was a requirement for a functional electron transport system. The transport system in Anabaena was previously shown to oxidize NADPH preferentially and appeared to be partially coupled to the phosphorylation of ADP. The possibility that the uptake of glucose 6-P in the dark was dependent on the ATP formed during the oxidation of NADPH was therefore investigated. In Figure 19 the effects of an uncoupler, FCCP (250 μM), and two energy transfer

Figure 18. The effect of electron transport inhibitors on the assimilation of glucose 6-P in the dark by Anabaena flos-aquae. The assimilation of glucose 6-P was followed as described in section D of the Materials and Methods. The radioactive assay contained cells equivalent to 250 µg chlorophyl, and 6.3 moles of glucose 6-P (specific radioactivity 0.16 µCi/µmole).

Control-Dark	(△)
2.5 mM SHAM	(●)
200 µM Rotenone	(■)
200 µM Antimycin A	(○)

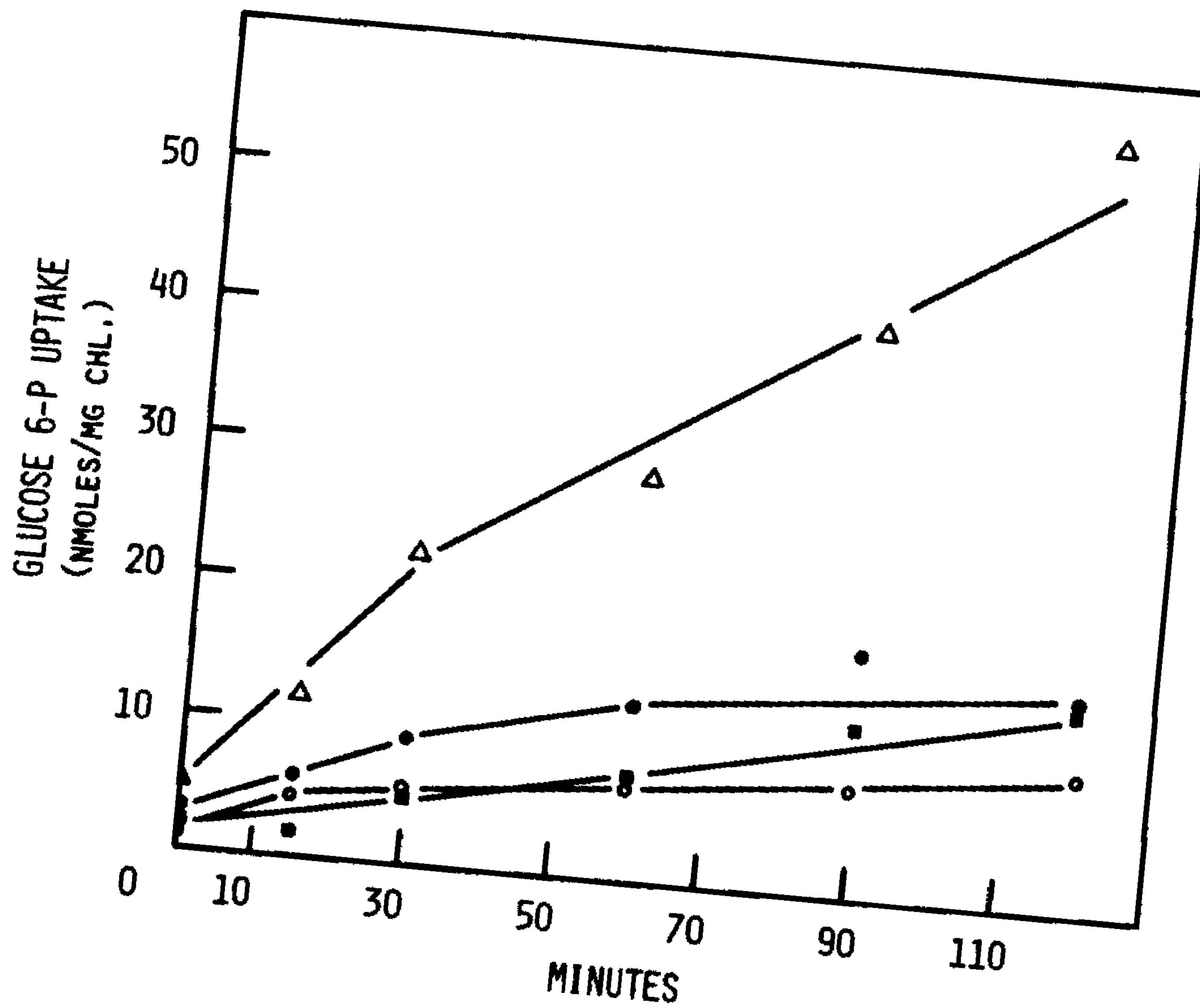
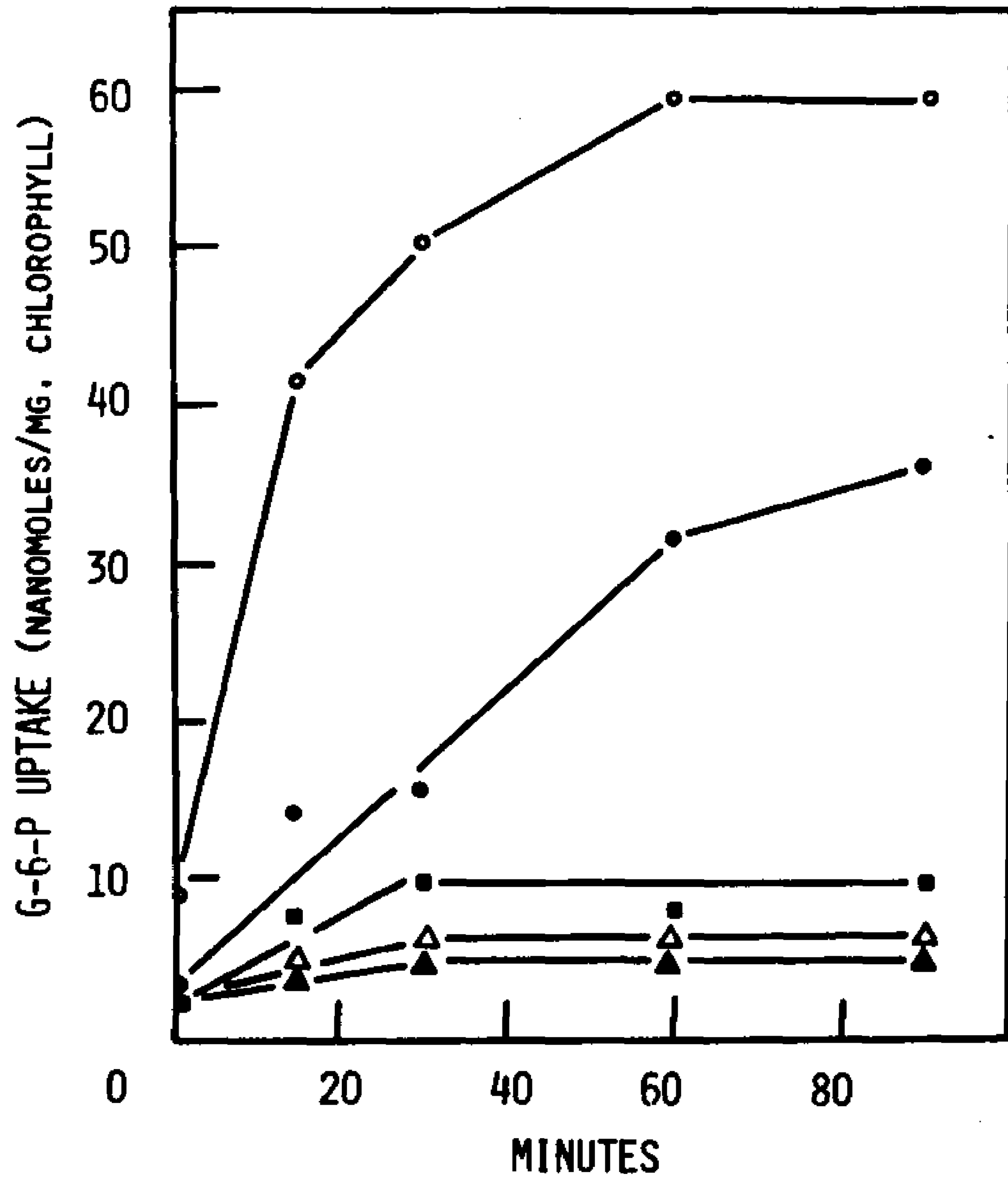

GLUCOSE 6-P UPTAKE
(NMOLES/MG CHL.)
50
40
30
20
10
0
10
30
50
70
90
110
MINUTES

Figure 19. The effects of FCCP, Oligomycin, and Phloridizin on the assimilation of glucose 6-P in the dark by *Anabaena flos-aquae*. The assimilation of glucose 6-P was followed as described in the Materials and Methods. The assay contained cells equivalent to 250 μg chlorophyl and 6.3 μmoles of glucose 6-P (specific radioactivity of 0.16 μCi/ μmole).

Control-Dark	(●)
250 μM FCCP	(▲)
100 μM Phloridizin	(△)
.025 mg Oligomycin	(■)
Control-Light	(○)


G-6-P UPTAKE (NANOMOLES/MG. CHLOROPHYLL)
60
50
40
30
20
10
0
20
40
60
80
MINUTES

inhibitors, oligomycin (0.5 mg/assay) and phloridizin (100 μM), are given. The energy uncoupler inhibited dark assimilation of glucose-6-P by about 72%, which indicated a coupling of the energy produced by the aerobic respiration of NADPH to glucose-6-P uptake.

Phloridizin (Izawa and Good, 1972), a potent antagonist of both Ca^{+2} and Mg^{+2} dependent ATPase activity, was previously demonstrated to inhibit the photomediated uptake of the sugar phosphate. From the results presented in this figure phloridizin also inhibited the dark assimilation of glucose-6-P by about 82%. Oligomycin, a known energy transfer inhibitor, is believed to inhibit the formation of ATP by acting at the site of the coupling mechanism (Lardy et al., 1958). As seen in Figure 19, oligomycin inhibited the initial rate of glucose-6-P accumulation by 40% but inhibited the final accumulation by 66%. These results indicated that the energy which was released during the oxidation of NADPH by the oxidase system of this organism was coupled to the formation of ATP, and this ATP could be utilized for the uptake of exogenous glucose-6-P in the dark.

Conclusion

The stimulatory effects that the various sugar phosphates have on the rate of CO_2 fixation in Anabaena 1444, when supplied to the cells in the dark were discussed in Section A of the Results and Discussion. The qualitative effects observed with the various sugar phosphates on the rate of CO_2 fixation were overall very similar to the results observed in the isolated spinach chloroplast system, except for the effects of glucose 6-P. The implications of these similarities between a chloroplast system and a blue-green alga could provide another piece of evidence in favor of the endosymbiotic theory of chloroplast evolution. Unfortunately like most of the other similarities between the two systems which have been pointed out as supporting this theory, it does not prove the endosymbiotic theory, nor disprove the evolutionary theory: it merely allows the endosymbiotic theory to exist. Finding that the blue-green algae and isolated chloroplasts respond to exogenously supplied phosphorylated compounds in a similar manner may merely mean that they both evolved from a common ancestor.

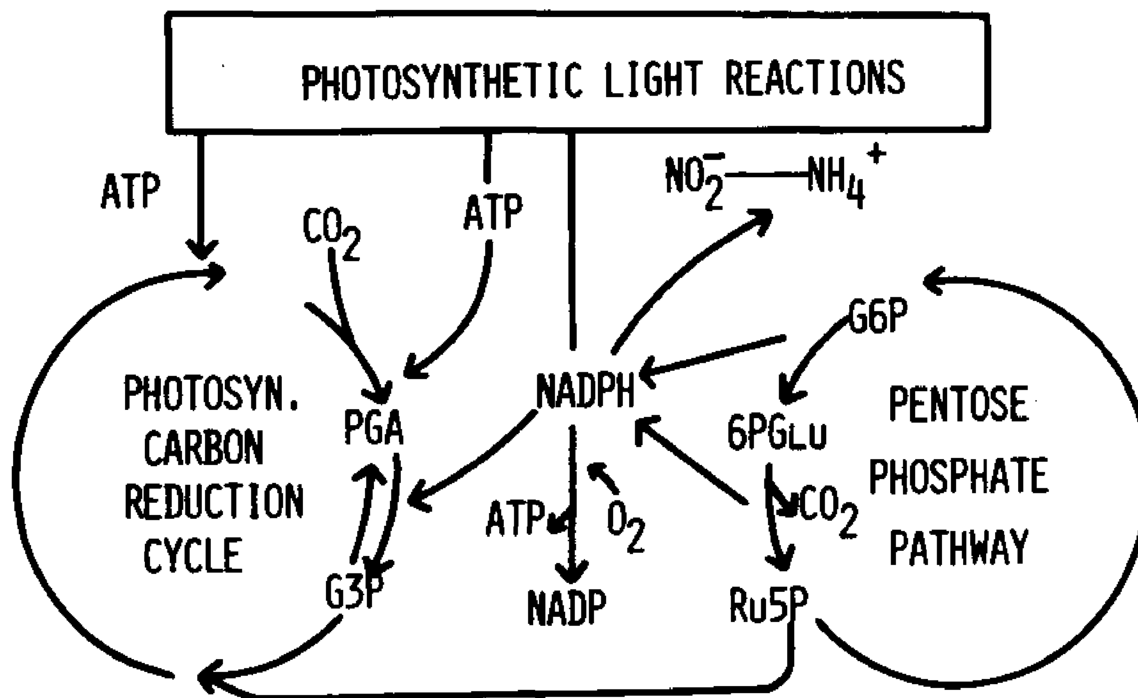
On the side of the evolutionary theory, there are chloroplasts and cells which do not fix CO_2 predominately by way of the Calvin cycle but use the C-4 (Hatch-Slack) pathway. There are no known blue-green algae

which autotrophically fix CO_2 predominately into C-4 acids. Perhaps more interesting on the biochemical level are the effects observed with glucose 6-P, which have not been observed in isolated chloroplast systems. The lack of effect by glucose 6-P and fructose 6-P on the rate of CO_2 fixation in isolated chloroplasts was believed to be the result of the impermeability of the chloroplast envelope to the hexose monophosphates. In the microorganisms such as _E. coli_ it was first generally believed that various phosphorylated compounds could not enter the cells intact but were first dephosphorylated (Lichenstein, 1960; Fraenkel _et al._, 1964). Winkler and others then described a specific system for the assimilation of the hexose monophosphates. The observation that glucose 6-P substantially stimulated the rate of CO_2 fixation and was capable of increasing the inhibition of CO_2 fixation caused by nitrite indicated that perhaps glucose-6-P was entering the cells as an intact molecule.

Glucose 6-P is a key intermediate in the carbohydrate metabolism of the blue-green algae: it plays a key role in glycogen synthesis, polysaccharide formation, and serves as a link between the Calvin cycle and the pentose phosphate pathway. The control of glucose 6-P turnover is also a key step in the regulation of the carbohydrate metabolism of these obligate photoautotrophs. It was therefore of great interest to investigate the possibility

of its being assimilated by Anabaena 1444 as an intact molecule. The data from the double label experiments and others (see Section C) indicated that glucose 6-P was being assimilated as an intact molecule and was not undergoing a dephosphorylation before entry into the cells. In diagram 1, the results obtained with glucose 6-P during light and dark CO_2 fixation are explained. As shown in this diagram, the exogenously supplied glucose 6-P can be oxidized by way of the pentose phosphate pathway when it is supplied in the dark period prior to the start of the experiment, and supplies NADPH and ribulose 5-P to the photosynthetic carbon reduction cycle. These then serve to increase the rate of CO_2 fixation by decreasing the lag period, and in addition the exogenously supplied glucose 6-P or a product of its oxidation possibly stabilizes a component in the blue-green algae needed to attain maximal rates of CO_2 fixation. This component might be an enzyme of the carbon reduction cycle, or a membrane component involved in energy transfer. This stabilization of a key component by glucose 6-P is based on the hypothesis by Schacter and Gibbs (1971) from work performed on CO_2 fixation in isolated chloroplasts. Though there is no direct proof for such a component in the blue-green

Diagram 1



algae, indirect evidence for stabilization or stimulatory components does exist in other systems, Schacter _et al_. (1971) found that Antimycin A at low concentrations (0.5-10 µM) increased the rate of CO_2 fixation, O_2 evolution and Pi esterfication in intact spinach chloroplasts. These effects were believed to be the result of Antimycin A binding to and affecting the outer membrane of the chloroplast which contained the enzymes involved in controlling the rate of CO_2 fixation. Wildner and Criddle (1969) have reported on a factor which is light activated (LAF) and which is capable of stabilizing the enzyme ribulose diphosphate carboxylase. This stabilization is reported to increase the rate of CO_2 fixation by 100%. In various C-4 and CAM (crassulacean acid metabolism) plants glucose 6-P (2 mM) has been shown to play an important role in photosynthetic metabolic regulation by activating the enzyme phosphoenolpyruvate carboxylase (Ting and Osmond, 1973). The activation by glucose 6-P is presumably the result of lowering the $K_{0.5}$ for phosphoenolpyruvate; glucose 6-P also reverses the substrate level inhibition of this enzyme by malic acid. It is therefore not unreasonable to suggest that glucose 6-P or one of the products of its oxidation may be involved in stabilizing an important factor involved in controlling the rate of CO_2 fixation in the blue-green algae.

When nitrite is present in the assay, the production of NADPH by way of the pentose phosphate pathway is envisioned as stimulating the reduction of nitrite to ammonia. The nitrite reductase complex responsible for this reduction has a relatively low K_M for NADPH (Hattori and Meyers, 1966). It therefore is an effective competitor with the carbon reduction cycle (glyceraldehyde 3-P dehydrogenase) for the reduced pyridine nucleotide. The increased production of NADPH by the oxidation of exogenously supplied glucose 6-P probably does not saturate the two enzyme systems; the NADPH is believed to be more effectively utilized by the nitrite reductase complex, resulting in the increased production of ammonia. As mentioned in section B, the ammonia can then feed back and inhibit the light reaction by acting as an uncoupler of photophosphorylation, or the ammonia can drain off carbon skeletons from the Calvin cycle by the stimulation of transamination, which would reduce the internal pool sizes of the Calvin cycle intermediates, and thus lower the observed rates of CO_2 fixation.

Enhancement of dark CO_2 fixation by glucose 6-P as shown in diagram 1 is believed to be the result of glucose 6-P oxidation in the dark by way of the pentose phosphate pathway and the coupling of the NADPH produced by this oxidation to ATP formation by the oxidase system. The NADPH, ribulose 5-P, and ATP then can enter the

reductive carbon cycle stimulating the rate of CO_2 fixation. This is in agreement with the results of Togasaki and Gibbs (1967) who found that enhanced dark CO_2 fixation in Anacystis and Chlorella was the result of two distinct processes. One was the carboxylation of the acceptor molecule RuDP, which was formed in the light, and the other was the reduction of the 3-phosphoglycerate, by NADPH which was generated in the dark.

After a brief period of illumination exogenously supplied glucose 6-P had no effect on the rate of CO_2 fixation in the light. As discussed in the previous section (A of the Results and Discussion), this was believed to be due to the buildup of the internal pool of glucose 6-P and the prevention of its oxidation by way of the pentose phosphate pathway (Pelroy and Bassham, 1972). The lack of an effect by the exogenously supplied glucose 6-P on CO_2 fixation has been shown not to be due to the inability of the glucose 6-P to be assimilated by the cells in the light, since the rate of assimilation of glucose 6-P in the light was two to three times the rate of assimilation in the dark. The enhanced uptake in the light was believed to be the result of increased energy production during photosynthesis as compared to the alga's aerobic respiratory abilities. In the light, the assimilation of glucose 6-P appeared to be linked to ATP production during cyclic electron flow. This

conclusion was based on the increased sensitivity of glucose 6-P assimilation to Antimycin A, an inhibitor of cyclic photophosphorylation, as opposed to DCMU, an inhibitor of non-cyclic photophosphorylation.

Further evidence for this hypothesis has been obtained by Mauro (1975) who has shown that photomediated glucose 6-P assimilation in Anabaena 1444 could be supported by a psuedo-cyclic process. If the cells were treated with DSPD, (which inhibits both non-cyclic and cyclic electron flow by preventing the reduction of ferredoxin, (Robinson et al., 1975; Simonis, 1973)) then the addition of ferricyanide removed electrons before photosystem one and a psuedo-cyclic photophosporylation was produced. The ATP produced during this psuedo-cyclic process supported the accumulation of glucose 6-P at a comparable level to the control. Further, Mauro found that if the cells were fully inhibited with 50 μM DCMU, and PMS was added to promote cyclic photophosphorylation the photomediated uptake of glucose 6-P was stimulated. These results support the contention that the uptake of glucose 6-P in the light required ATP which was probably derived from the cyclic electron transport system.

The assimilation of glucose 6-P in the dark has also been shown to be energy dependent. The lower levels of glucose 6-P which were assimilated in the dark are indicative of the lower amounts of ATP available during

dark respiration. It seems very probable that the uptake of glucose 6-P in the light and dark occur by the same mechanism and that the difference in levels of substrate assimilated is related to the difference in energy production in the light and dark in these algae.

The dark uptake of glucose 6-P, (as shown in section E) was sensitive to various electron transport inhibitors, (Antimycin A, rotenone, and SHAM) and to energy transfer inhibitors, and uncouplers as (phloridizin, oligomycin, and FCCP). These results, besides indicating that the uptake of glucose 6-P in the dark was energy dependent, also indicated that the electron transport system in the blue-green alga was coupled to ATP production. These results are in agreement with the findings of Leach and Carr (1970) who performed *in vitro* studies on the oxidase system of *Anabaena variabilis*. The electron transport inhibitors chosen here were based on the work of Goldberg and McGowan (1975) on the NADPH oxidase system in *Anabaena* 1444. The use of SHAM as an inhibitor was based on the finding that cyanide had no inhibitory effect on NADPH oxidation on the *in vitro* system from *Anabaena* 1444; the ability of SHAM to inhibit the oxidase system is suggestive of an o-type cytochrome as the terminal electron acceptor and not an $a\text{-}a_3$ type cytochrome. This belief was based on the work of Hill *et al*. (1973) who used a protozoal system to study the effect of SHAM on

terminal oxidases, and on the work of Schobaum _et al._ (1971) on the effects of hydroxamic acids on the cyanide insensitive pathway in plant mitochondria. The inhibition caused by Antimycin A and rotenone indicated that there may be two flavoproteins in the electron transport system, one between cytochrome _b_ and cytochrome _c_ and the other flavoprotein between the pyridine oxidoreductase and cytochrome q. Goldberg and McGowan (1975) have also shown that the addition of flavin in the form of FMN and FAD causes a large stimulation of O_2 uptake and NADPH oxidation in the _in vitro_ oxidase system; this may indicate either a very low level of endogenous flavin or a loss of it during the extraction of the oxidase system.

The uptake of glucose 6-P in the light and dark is therefore believed to be an energy dependent process; in the light the ATP is believed to be derived from cyclic photophosphorylation and in the dark derived from the NADPH oxidase system. The next logical question is -- Can the glucose 6-P which was accumulated by the cells support either enhanced growth in the light or at least low rates of growth in the dark (chemoheterotrophic)? No stimulation of growth has been found either in the light under mixotrophic growth conditions, or in the dark under heterotrophic growth conditions.

The finding that glucose 6-P did not support growth under chemoheterotrophic conditions even to limited extent was at first surprising. Glucose 6-P was known

to be assimilated by the cells, thereby circumventing one of the major restrictions (the hexokinase reaction) in the utilization of free sugars such as glucose or fructose. Hexokinase has been demonstrated to be present in very low amounts (Pelroy, Rippka, and Stanier, 1972; Carr and Whitton, 1973) and therefore was believed to be the rate limiting step in the utilization of the sugars for growth. The fact that glucose 6-P did not support chemoheterotrophic growth may indicate another block or rate limiting step in the utilization of hexose sugar or the hexose 6-P such as glucose 6-P. In the turn-over data presented it was shown that only a small percentage of the total glucose 6-P taken up was oxidized by way of the pentose phosphate pathway. This limited turnover of glucose 6-P was not believed to be due to low levels of the pentose phosphate pathway enzymes, since these enzymes have been shown to be present in large amounts (Pelroy, Rippka and Carr, 1972). One of the hypotheses put forth based on the work of Goldberg and McGowan (1975) and Leach and Carr (1970), was that the NADPH oxidase system was actually responsible for the limited turnover of the glucose 6-P in the dark. During the oxidation of glucose 6-P in the dark, the NADPH generated was not reoxidized at a sufficient rate by the oxidase system to maintain a rapid turnover of glucose 6-P nor did it allow for sufficient ATP production to support active growth.

One of the possible mechanisms by which the NADPH oxidase system was relegated to a minor or rate limiting role in the blue-green alga metabolism might be due to relative reduction in the amount of flavins (FMN or FAD). As Goldberg (1975) showed the addition of flavin stimulates the NADPH oxidase system to a very large degree; this reduction of flavin content may therefore be one of the primary processes involved in the evolution of an obligate photoautotrophic physiology.

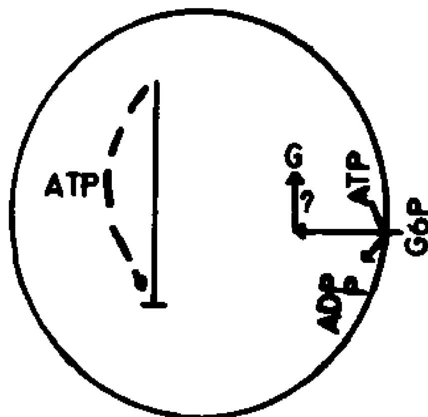
The lack of any stimulation of growth under mixotrophic conditions in the light indicates the overwhelming preference for CO_2 as the carbon source: this may be due as Carr (1973) suggested, to the inability of these algae to adjust their enzyme levels in order to take full advantage of organically enriched environments. One project that would be fruitful would be to isolate a regulatory mutant in these algae, of the enzyme glucose 6-P dehydrogenase which can oxidize glucose 6-P in the light. As discussed previously glucose 6-P dehydrogenase is the regulatory point in the pentose phosphate pathway, and this regulation is responsible for the prevention of the oxidation of glucose 6-P in the light. It is believed, Grossman and McGowan (1975), that ATP and NADPH produced during photosynthesis are the effectors of this inhibition. Therefore a regulatory mutant with an enzyme unaffected by ATP and NADPH should be capable of oxidizing glucose

6-P under conditions of high light and low CO_2, thereby stimulating growth.

The overall question now becomes: what is the function of the glucose 6-P uptake system? As discussed, it is an energy dependent system, but unlike the glucose 6-P transport system in *E. coli*, it cannot support active growth. The glucose 6-P transport system in *E. coli* is an inducible system (Winkler, 1973) coupled to energy produced during oxidative respiration (Winkler, 1973). This system adds versatility to the heterotrophic metabolism of this enterobacterium. When *E. coli* cells find themselves in an enriched environment for glucose 6-P, they can respond by increasing the uptake and utilization of this hexose 6-P and thereby increase their growth rate. Winkler (1973) has, in fact, shown that inducible hexose 6-P uptake systems similar to *E. coli* are widespread among bacteria. The findings that photoautotrophically grown cells of *Anabaena* 1444 are able to assimilate glucose 6-P (although the system does not appear to be inducible) offers to the investigator an evolutionary and biochemical challenge regarding the function of this sugar phosphate uptake system; the cell is expending metabolically produced energy in assimilating this molecule and is seemingly deriving very little in return. In order to rationalize this situation a hypothetical evolutionary scheme for the blue-green algae has been developed and is shown in diagram 2.

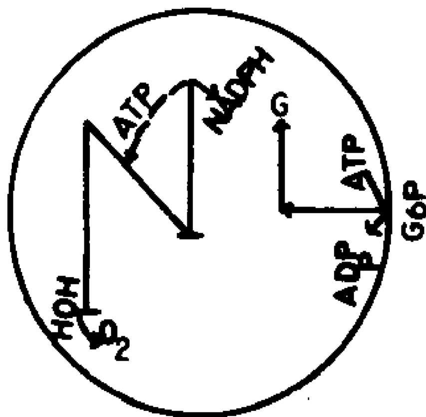
DIAGRAM 2

ANAEROBIC



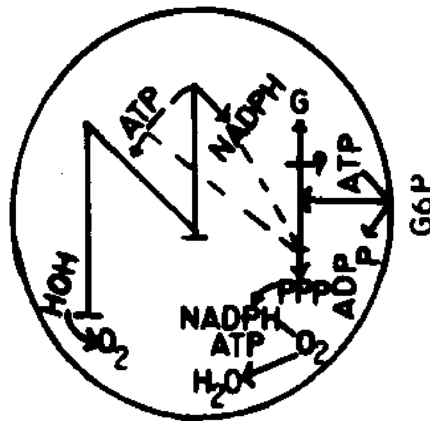
1

AEROBIC?



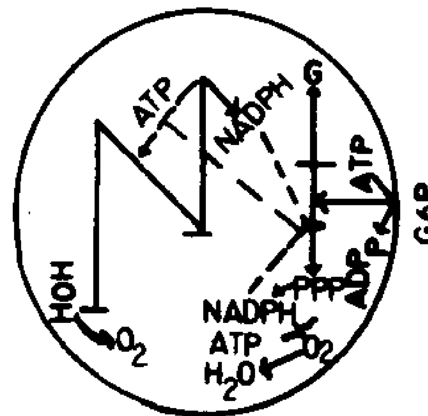
2

AEROBIC



3

AEROBIC



4

This scheme is based to some extent on the ideas of Uzell and Spolsky (1974). In 2a, the first step in the evolution of a photosynthetic organism is shown as the evolution of photosystem I, which is capable of cyclic photophosphorylation. This photosystem evolved during the anaerobic phase of the earth's atmosphere. During this stage of the alga's evolution the glycolytic pathway may also have evolved. It is believed that the glycolytic pathway was one of the most primitive pathways common to all cell types and was capable of operating under anaerobiosis. It is postulated that at this time, the energy produced during cyclic photophosphorylation was at least in part coupled to the assimilation of glucose 6-P from the environment. The glucose 6-P so assimilated was used primarily for biosynthetic purposes or oxidized by way of the glycolytic pathway.

In diagram 2b, the environment became aerobic, a change believed to be directly related to the evolution of photosystem II by the evolving prokaryotic algae. This photosystem, capable of photolysis and O_2 evolution, was believed to be responsible for the aerobic atmosphere. The existence of the glycolytic pathway in these alga during this time was fairly certain and the assimilated glucose 6-P from the medium was used both for biosynthetic purposes and for oxidative metabolism via the glycolytic pathway. Cyclic photophosphorylation was still believed

to be responsible for energizing the assimilation of glucose 6-P from the environment.

In diagram 2c, the environment was believed to be fully aerobic with the prokaryotic algae now able to photosynthesize with two photosystems and fix CO_2 by way of the Calvin cycle. The exogenous glucose 6-P could be respired by the glycolytic pathway or the pentose phosphate pathway. With the evolution of the pentose phosphate pathway, the NADPH oxidase system had to evolve to regenerate the electron acceptor. The regulation of glucose 6-P oxidation in the light probably existed at this time, with the turnover of glucose 6-P in the dark coupled to the oxidase system allowing for full heterotrophic growth. As indicated in this diagram the oxidase system was fully coupled and there was a full complement of flavins in the transport system at this time so that there was no restriction in the utilization of glucose 6-P for growth. At this point in the evolution of these algae the glycolytic pathway became relegated to the minor role in the sugar oxidation that it now plays. This relegation may have occurred by a decrease in the synthesis of the enzyme phosphofructokinase. At this point in time the primative prokaryotes were able to photosynthesize and grow autotrophically and were capable of active accumulation of glucose 6-P for aerobic respiration (heterotrophic growth).

In diagram 2d, the final stage in blue-green algae evolution is presented. Here glucose 6-P was actively accumulated and coupled to the ATP produced by photosystem I activity. As shown, the glycolytic pathway is almost non-functional and the pentose phosphate pathway is active to some degree in the dark with its activity being limited by the NADPH oxidase system, which has been relegated to a minor role in these algae's metabolism. At this point these prokaryotes were well on their way to becoming obligate photoautotrophs. The uptake system that has been described appears to be a remnant of a system which at first served to supply substrate for biosynthetic purposes and then provided substrate for active physiological respiration. Now, however, the transport system plays only a minor role, being important in those few filamentous or unicellular forms which can grow photoheterotrophically (where the uptake system once again would provide substrate for biosynthetic purposes).

Numerous hexose monophosphates, such as glucose 6-P, and fructose 6-P, can be assimilated by a variety of bacteria by means of an inducible hexose phosphate transport system (Winkler, 1973). The widely held assumption that anions, such as phosphates or sulfates, were not readily assimilated by intact cells was disproved by the demonstration of this inducible transport system in bacteria. The finding that a blue-green alga, such

as *Anabaena flos-aquae* 1444, which was believed to be an obligate photoautotroph, also has a transport system capable of assimilating exogenous glucose 6-P suggests a closer examination of the general accepted belief that phosphorylated compounds are not assimilated by intact cells. Cohen (1975) in a commentary on the use of various nucleosides in cancer chemotherapy, made the point that the pervasive influence of the exclusion rule must be overcome. It has been shown (Cohen, 1974; Plunket, 1975) that phosphorylated compounds as nucleotide analogs are taken up and metabolized by eukaryotic cells. Until these demonstrations of nucleotide uptake it was believed that only the nucleosides were incorporated into cells. It is therefore hoped that with these demonstrations that intact cells can incorporate various phosphorylated compounds, the generalized idea of non-assimilation of phosphorylated compounds will be disproved. It is hoped that future investigations into the incorporation of phosphorylated compounds and other anions in various systems will be undertaken, and from such studies the possibility of finding more efficient ways of examining *in vivo* cellular metabolism and regulation may emerge.

APPENDIX A

Composition of Culture Medium-Bristol's Solution.

Starr, R. C. 1964. The culture collection at Indiana University. American Journal of Botany. 51; 1013-1044.

$NaNO_3$	750	mg/l
$CaCl_2 \cdot 2H_2O$	25	mg/1
K_2HPO_4	75	mg/1
KH_2PO_4	175	mg/1
$MgSO_4 \cdot 7H_2O$	75	mg/1
NaCl	25	mg/1
EDTA	50	mg/1
KOH	31	mg/1
$FeSO_4 \cdot 7H_2O$	4.98	mg/1
H_3BO_3	1.14	mg/1
$ZnSO_4 \cdot 7H_2O$	8.8	mg/1
MoO_3	0.71	mg/1
$CuSO_4 \cdot 5H_2O$	1.5	mg/1
$Co(NO_3) \cdot 6H_2O$	0.49	mg/1
$MnCl_2 \cdot 4H_2O$	1.44	mg/1
Soil extract	1.6	gm/1

APPENDIX B

Composition of Wood's GW_3 Chromatographic Solvent.
Wood, T. 1961. A procedure for the analysis of acid-soluble phosphate compounds and related substances in muscle and other tissue. J. Chromatography. 6; 142-154.

n-Butanol	64	ml
n-Propanol	32	ml
Acetone	40	ml
30% w/w Trichloro-acetic acid	24	ml
80% w/v formic acid	40	ml
EDTA	0.6	gm/200 ml

APPENDIX C

Wilkinson and Davies Chromatographic Solvent.

As Communicated to the author by Dr. Dan King.

Triethylamine	10 ml
Acetone	160 ml
H_2O	30 ml

APPENDIX D

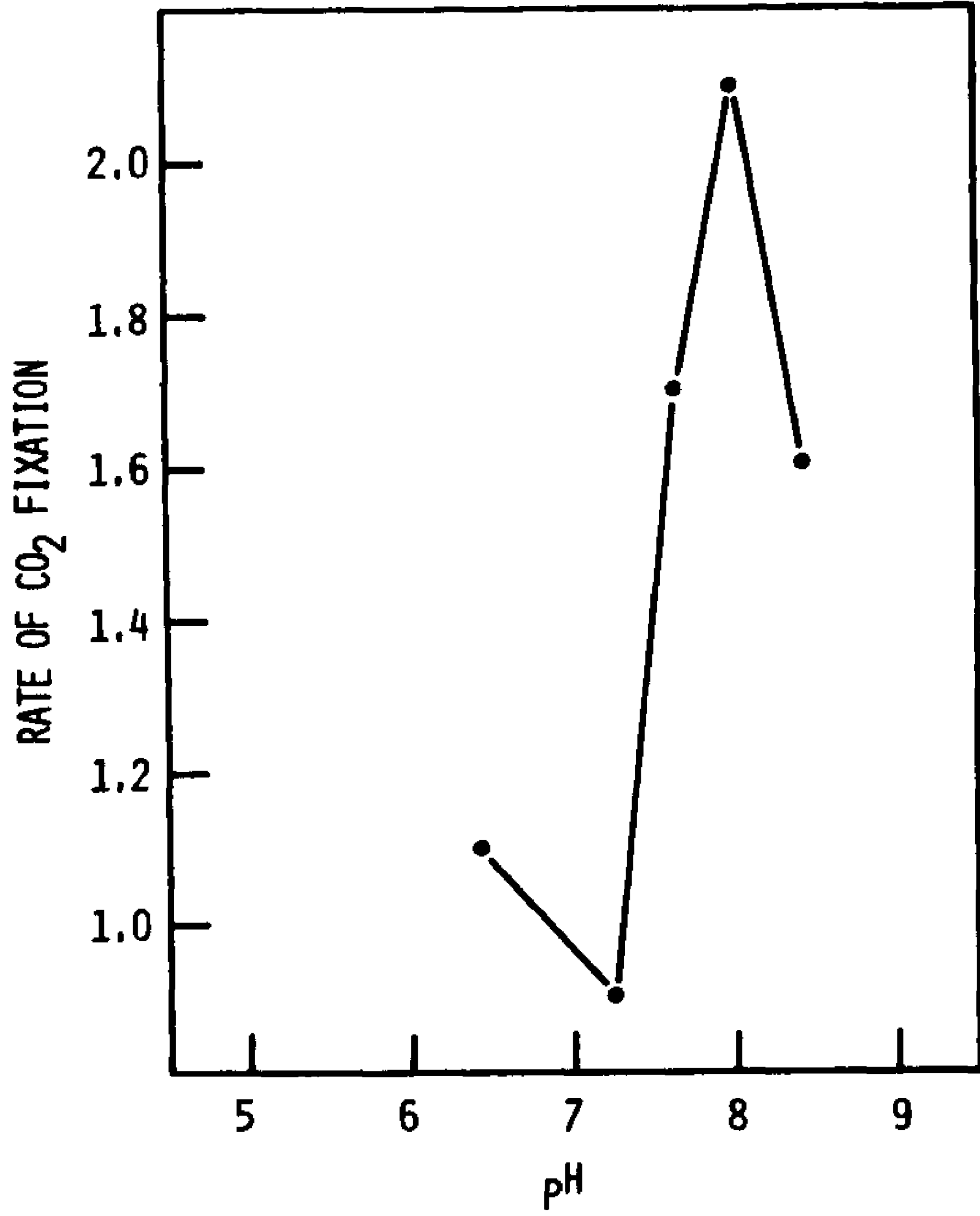
Phosphate Indicator Spray.

Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate, and phosphatases. Methods in Enzymology. VII; 115-118.

60% w/w Perchloric acid	5 ml
1 N HCl	10 ml
4% w/v Ammonium molybdate	25 ml
H_2O	60 ml

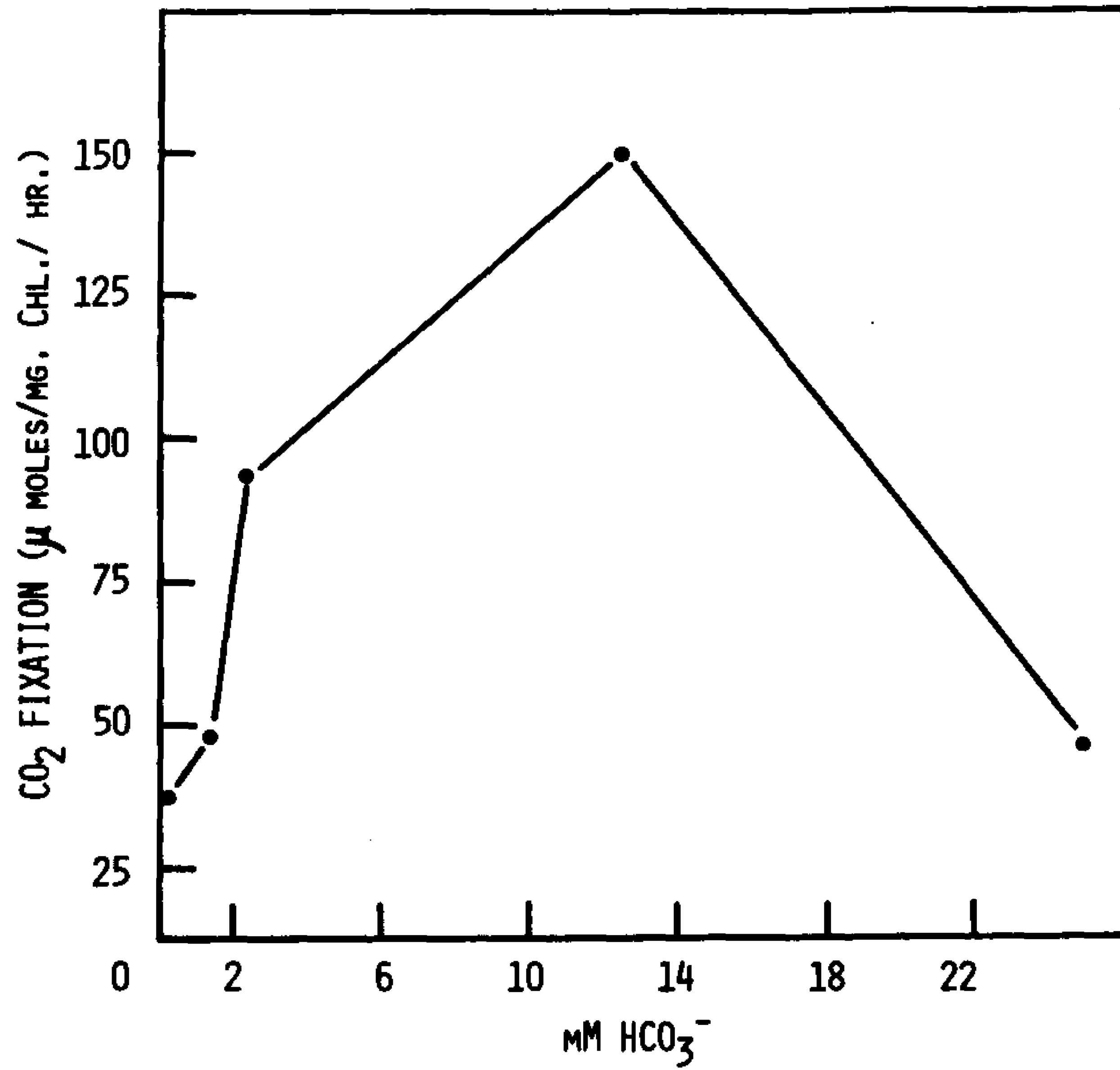
APPENDIX E

The effect of pH on the rate of CO_2 fixation in *Anabaena flos-aquae*. The experiments were performed as described in the Materials and Methods section C, except that the HEPES (25 mM) was adjusted to the indicated pH.


RATE OF CO_2 FIXATION
2.0
1.8
1.6
1.4
1.2
1.0
5
6
7
8
9
pH

APPENDIX F

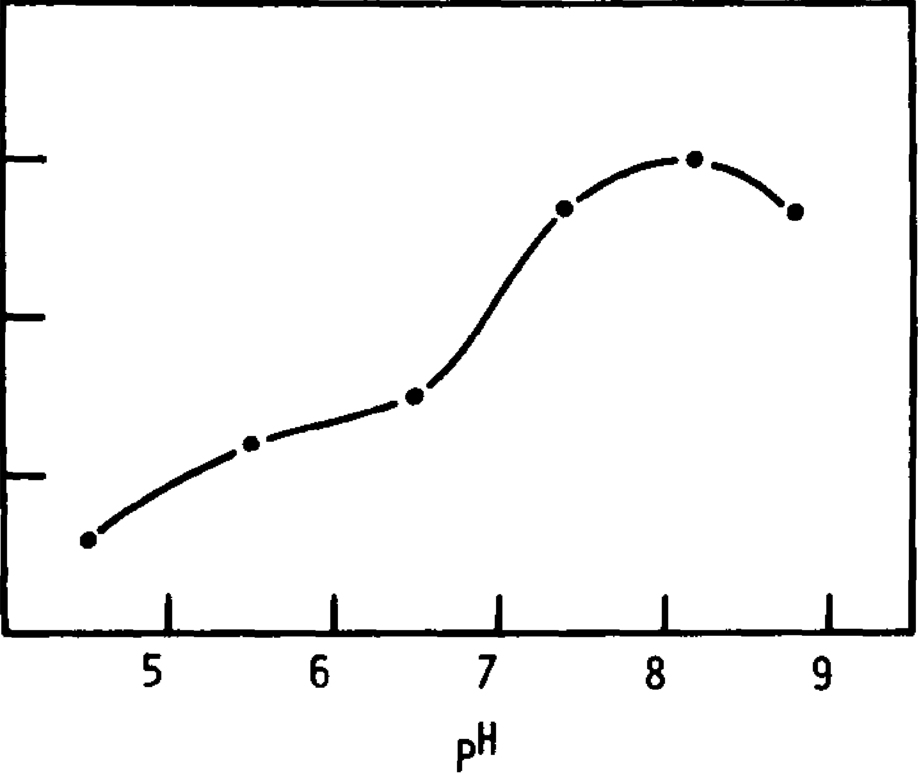
The effect of the HCO_3^- concentration on the rate of CO_2 fixation in *Anabaena flos-aquae*. The experiments were performed as described in Materials and Methods section C.



CO2 FIXATION (μ MOLES/MG. CHL./ HR.)
150
125
100
75
50
25
0
2
6
10
14
18
22
mM HCO3-

APPENDIX G

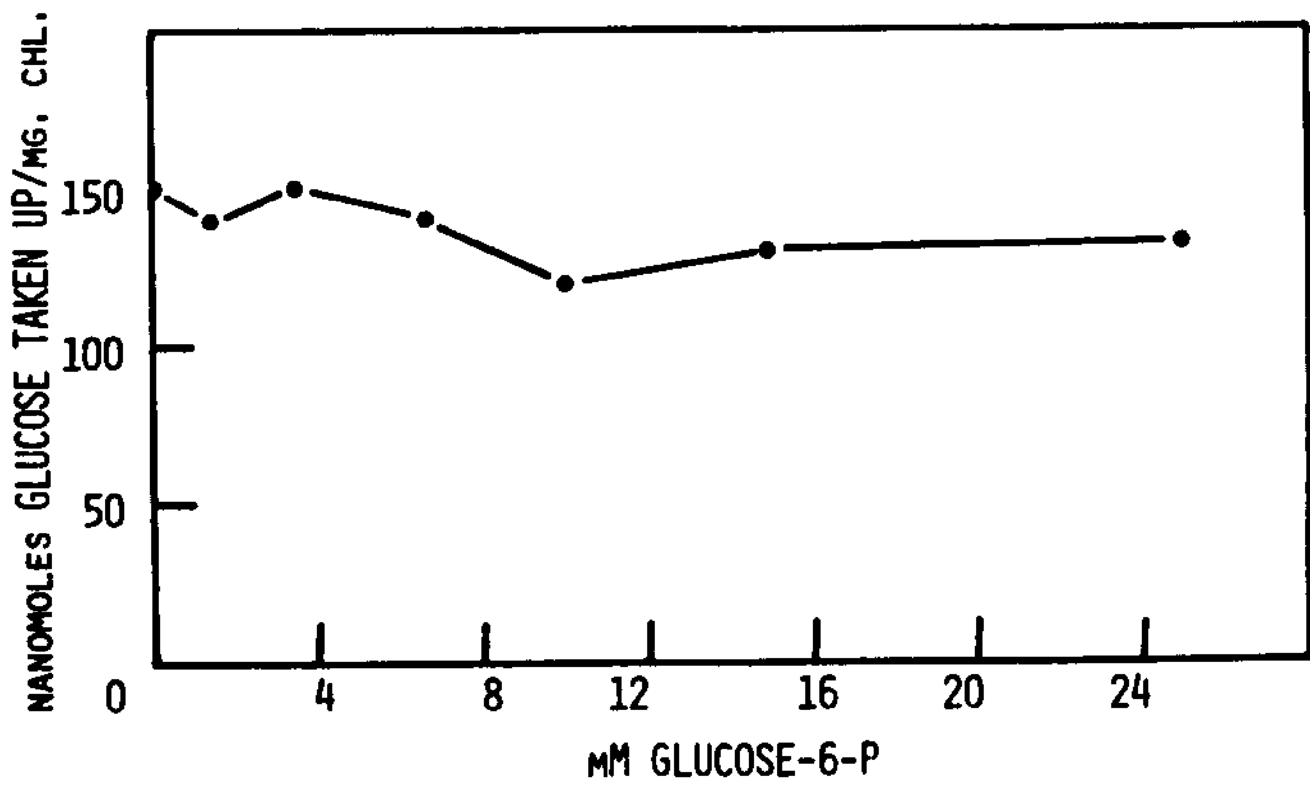
The effect of pH on the rate of glucose 6-P uptake in the light. The uptake experiments were performed as described in the Materials and Methods, except that the Bristol's solution was adjusted to the indicated pH using HCl or KOH.



NANOMOLES GLUCOSE-6-P TAKEN UP/MG.CHL/HR.
25
50
75
5
6
7
8
9
pH

APPENDIX H.

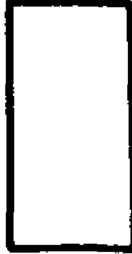
The effect of glucose 6-P on the uptake of glucose by *Anabaena flos-aquae*. The assimilation of glucose was followed as described in the Materials and Methods section D. The 2 ml incubation mixture contained cells equivalent to 250 μg chlorophyll, 6.3 μmoles of glucose (specific radioactivity 0.16 μCi per μmole), and varying amounts of ^{12}C-glucose 6-P.


NANOMOLES GLUCOSE TAKEN UP/MG. CHL.
0
50
100
150
4
8
12
16
20
24
mM GLUCOSE-6-P

APPENDIX I

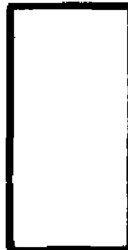
Chromatographic separation of various assimilated phosphorylated metabolites. The extraction and analysis of the assimilated products were performed as described in the Materials and Methods section F.

DEOXY GLUCOSE-6-^{32}P

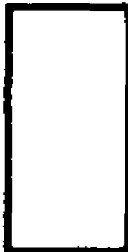


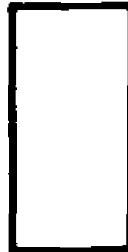
^{14}C GLUCOSE-1-P

LIGHT ^{14}C GLUCOSE



DARK ^{14}C GLUCOSE





ORIGIN

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