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Uptake and Utilization of Sugar Phosphates by *Anabaena flos-aquae*[1]

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ABSTRACT

The effect of various sugar phosphates on CO_2 fixation in *Anabaena flos-aquae* was investigated and found to be very similar to that found for isolated spinach chloroplasts. One exception, glucose 6-phosphate, has a stimulatory effect on CO_2 fixation in *Anabaena* but not in isolated chloroplasts.

Further examination of the role of glucose 6-phosphate metabolism in *Anabaena* indicates that: (*a*) this sugar phosphate can be taken up; (*b*) its uptake is greater in the light than the dark; (*c*) turnover of glucose 6-phosphate is inhibited in the light; and (*d*) glucose 6-phosphate can support dark CO_2 fixation. These results are discussed with reference to photosynthesis-related control of glucose 6-phosphate metabolism and the role of glucose 6-phosphate as a source for reducing equivalents and ATP in blue-green algae.

The generally obligate nature of photoautotrophic growth in the blue-green algae has prompted the investigation of the biochemical basis for this growth habit. Although their mode of photosynthesis seems to be essentially identical to that of eucaryotic organisms (9), a blocked tricarboxylic acid cycle and a limited glycolytic cycle, due to the absence or low levels of phosphofructokinase (11, 12, 16, 18), suggest that carbon flow in these organisms is either by a catabolic pentose phosphate shunt, as shown by Cheung and Gibbs (5), or by direct utilization of triose produced during photosynthetic carbon reduction. The questionable functioning of glycolysis and the tricarboxylic acid cycle, and the concomitant lack of ATP synthesis in the dark, might be the reason for limited growth on glucose or acetate (11, 12, 16).

Recently, Pelroy and Bassham (13-15), have shown that turnover of glucose-6-P is restricted in the light and that rapid turnover occurs in the dark. Grossman and McGowan (6) have investigated the kinetics of glucose-6-P dehydrogenase in *Anacystis* and *Anabaena* and have determined regulation of the enzyme's catalytic activity by pH, NADPH, and ATP. These data suggest that if glucose-6-P could be assimilated in the dark, its turnover via the pentose phosphate pathway should go unhindered. However, it is generally believed that sugar phosphates are not assimilated by whole algal cells, or that if they are, they are first dephosphorylated at the cell membrane.

In this study we present data which suggest that some sugar phosphates can be assimilated by *Anabaena flos-aquae*, particularly glucose-6-P, and that they can be used as probes of the mechanism of autotrophic growth.

MATERIALS AND METHODS

Glucose-6-P monosodium salt, 6-P-gluconate trisodium salt, fructose-1,6-diP trisodium salt, fructose-6-P disodium salt, ribose-5-P disodium salt, 3-P-glycerate monosodium salt, and HEPES were obtained from Sigma Chemical Co. Omnifluor was purchased from New England Nuclear and Triton X-100 was a product of Rohm and Haas. [UL-^{14}C]Glucose-6-P and D[1-^{14}C]glucose were obtained from ICN Pharmaceuticals Inc. [1-^{14}C]Glucose-6-P was a gift of M. Belsky and was originally obtained from New England Nuclear. γ-[^{32}P]ATP was obtained from New England Nuclear. All radioactive metabolites were determined to be chromatographically pure by co-chromatography with standards. $Ba^{14}CO_3$ was purchased from New England Nuclear and converted to $NaHCO_3$ by trapping in 3 N NaOH the CO_2 released by treatment with lactic acid. All other chemicals were reagent grade.

Anabaena flos-aquae (Bloomington 1444) was obtained from the Indiana culture collection and grown in 2-liter Erlenmeyer flasks in Bold's basal medium plus soil extract (19). The flasks were shaken on a gyratory shaker at 150 rpm and illuminated from above by a bank of Sylvania Gro-Lux lights to give a final illumination of 5×10^5 ergs cm^{-2} sec^{-1} at the flask base. The cultures were continuously monitored for contamination by spreading aliquots on nutrient agar plates and incubating the plates for at least 4 days at 35 C. The cultures were harvested by centrifugation at 10,000*g* for 20 min. The pelleted cells were washed once in 50 mM HEPES buffer at pH 8, recentrifuged, and resuspended in the same buffer to give a cell density of 150 to 200 mg Chl $\cdot$ ml^{-1}. Chl was determined by the procedure of Arnon (1).

Carbon dioxide fixation was performed in test tubes (13 × 100 mm) with constant mixing provided by a small Teflon-coated magnetic stir bar. The tubes were immersed in a Plexiglas constant temperature bath at 25 C. Illumination of 10^7 ergs cm^{-2} sec^{-1} at the tube surface was provided by opposing bands of General Electric 75 w reflector spot bulbs. Unless otherwise stated, the 2.5-ml assays contained cells equivalent to 150 μg Chl, 25 μmol HEPES (pH 8), and 25 μmol $NaHCO_3$ (0.66 μCi/μmol). The assay solution minus the $NaHCO_3$ was incubated for 5 min in the dark followed by a further 5-min dark incubation with the $NaHCO_3$. Initiation of photosynthesis was achieved by illumination. Samples of 0.1 ml were removed at timed intervals and placed into 0.04 ml of concentrated formic acid. An aliquot of the acidified sample (25 or 50 μl) was placed on a planchet, dried, and counted on a Nuclear-Chicago gas flow counter. Fixation rates were calculated in the period between 2 and 8 min, the portion of the time course which gave the highest linear rate for all samples.

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Uptake of glucose or glucose-6-P was measured by removing a 0.1-ml aliquot from a cell suspension incubated in 1 ml of Bold's basal medium supplemented with radioactive metabolite. The cells were mixed with 6 ml of fresh medium, pelleted by centrifugation, resuspended in 6 ml of medium, repelleted by centrifugation, resuspended in 3 ml of medium, and isolated by filtration onto 2.4-cm Whatman GF/C glass fiber filters. The filters, containing the cells, were then adhered to planchets and the radioactivity determined in a gas-flow counter.

Turnover of absorbed metabolites was measured by trapping the CO_2 released by the cells. A side arm test tube (16 × 100 mm) was fitted with serum caps and a well containing a piece of fluted filter paper saturated with 2 N KOH. The 1-ml incubation mixtures consisted of cells suspended in Bold's basal medium supplemented with metabolite. At the end of the incubation period, 0.5 ml of 80% formic acid was introduced through the side arm and 0.5 hr was allowed for CO_2 trapping to occur. The filter papers were then removed from the wells and radioactivity determined in a scintillation cocktail of Omnifluor, Triton X-100, and toluene (0.25:75:125 w/v/v).

The preparation of doubly labeled glucose-6-P was achieved by mixing commercial [^{14}C]glucose-6-P and glucose-6-^{32}P. The glucose-6-^{32}P was generated in a reaction containing γ-[^{32}P]ATP, glucose, and hexokinase. The reaction was allowed to proceed for 3 hr, lyophilized, and chromatographed on Whatman 3MM paper in Wood's GW3 solvent (23). The glucose-6-P was eluted with water and rechromatographed in a solvent of triethylamine, acetone, and water (1:160:10, v/v/v; (D. King, personal communication). The glucose-6-P was eluted from this chromatogram with water, lyophilized, and used as an aqueous solution.

RESULTS AND DISCUSSION

Our studies of *Anabaena* 1444 indicate that added sugar phosphates have effects on CO_2 fixation similar to those observed for isolated chloroplasts (2, 4, 17). In isolated chloroplasts, several investigators have observed a reduction in the lag period of CO_2 fixation by certain sugar phosphates, particularly the pentose monophosphates (2, 4, 17). In several experiments, we observed a significant alteration in the rate of CO_2 fixation in the presence of a variety of relevant phosphorylated sugars. There was, however, no appreciable diminution of the lag period.

Schacter *et al.* (17) classified the various sugar phosphates into three groups depending on their effects on CO_2 fixation and O_2 evolution in spinach chloroplasts. In their system, fructose-1,6-diP, glyceraldehyde-3-P, and ribose-5-P are class I compounds, "giving maximal stimulation" of CO_2 fixation "and reversal" of some inhibitor effects. Fructose-6-P and glucose-6-P are class II metabolites, "giving slight effects"; P-glycerate is a class III compound, its effect being quite variable depending on the chloroplast preparation and concentration. Our observations on the effects of these compounds on CO_2 fixation in *Anabaena* are, with few exceptions, similar. In *Anabaena*, fructose-1,6-diP becomes inhibitory to CO_2 fixation if a 10-min illumination period precedes the addition of the HCO_3 and fructose-1,6-diP.

One significant difference between the effects of sugar phosphates on isolated chloroplasts and blue-green algae is that glucose-6-P, which has only a slight stimulatory effect on chloroplast CO_2 fixation, markedly stimulates in *Anabaena*. Glucose and Pi were without effect in these experiments.

Our purpose in these studies is not an attempt to evaluate the mechanism(s) of the many sugar phosphate effects on photosynthesis; rather, it is to determine whether exogenously added sugar phosphates can be utilized as probes of the autotrophic physiology of the blue-green algae. Our CO_2 fixation studies suggested the efficacy of this approach. The stimulation of CO_2 fixation in *Anabaena* by glucose-6-P is promising since this compound has been implicated as an important branch point intermediate in blue-green algae, with its turnover being controlled by some product(s) of photosynthesis (6, 16). Our goal, therefore, is to demonstrate that glucose-6-P enters *Anabaena* cells intact, turns over in a manner consistent with previous studies on *in vivo* pool size changes (8, 13), and affects several physiological functions.

Perturbation of Physiological Functions. Since it is known that, in *Aphanocapsa*, the glucose-6-P pool increases in the light and decreases in the dark (13), it is not surprising to find that glucose-6-P stimulates CO_2 fixation in cells which have been maintained in the dark for a period prior to measurement of photosynthetic CO_2 fixation. Figure 1 shows a saturation curve for glucose-6-P stimulation of CO_2 fixation in *Anabaena* cells maintained in darkness for at least 10 min prior to the measurement of fixation. This stimulation is not observed in cells which have been illuminated for 10 min prior to the measurement of fixation (Fig. 1). We envision that the glucose-6-P stimulation is due to some product(s) of glucose-6-P turnover which is limiting in the functioning of the Calvin cycle.

A second physiological function which is affected by exogenously added sugar phosphates is the inhibition of CO_2 fixation by nitrite. At high concentrations nitrite inhibits CO_2 fixation (50% inhibition at 33 mM) probably by competing with the photosynthetic carbon reduction cycle for reducing equivalents at the level of ferredoxin. Other possible mechanisms for nitrite inhibition are toxic effects of ammonium ion and/or hydroxylamine, and the promotion of amination reactions (such as glutamine formation) which could deplete the Calvin cycle carbon and ATP pools. Inasmuch as the blue-green algal nitrite reductase utilizes reduced ferredoxin, the oxidation of glucose-6-P might partially overcome the inhibition by nitrite. However, as illustrated in Figure 2, the opposite result was obtained. Of all of the sugar phosphates tested, only glyceraldehyde-3-P (at 4 mM) and glucose-6-P (at 7.5 mM) had any effect, and this was to enhance the inhibitory effect of nitrite. These results further demonstrate a physiological perturbation by exogenous sugar phosphates; however, we cannot offer a mechanism at present.

A third piece of evidence suggesting physiological effects produced by added sugar phosphates is the stimulation by glucose-6-P (Fig. 3) of low but measurable rates of CO_2 fixation in the dark. We chose to measure dark CO_2 fixation because it would specifically require both NADPH and ATP. Togasaki and Gibbs (20) have previously demonstrated a low, but measurable, level of dark CO_2 fixation in *Tolypthrix*. Our results indicate that if cells are preilluminated, dark CO_2 fixation is significant as compared to the dark control. However, if the cells are maintained in the dark and exogenous glucose-6-P is supplied, dark CO_2 fixation occurs at a rate 4-fold higher than that following preillumination.

Our results strongly suggest that exogenous glucose-6-P and perhaps some other phosphorylated intermediates can be used as

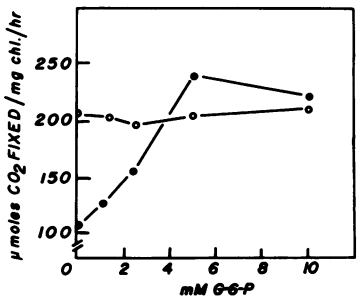

FIG. 1. Effect of glucose-6-P on the rate of CO_2 fixation by *A. flos-aquae*. Measurements of CO_2 fixation were performed as described under "Materials and Methods." The cells were either illuminated for 10 min prior to the addition of the $NaH^{14}CO_3$ and glucose-6-P (○), or the cells were maintained in darkness until the addition of $NaH^{14}CO_3$ and glucose-6-P (●).

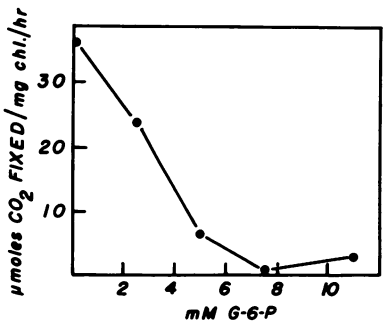

FIG. 2. Enhancement by glucose-6-P of the inhibition of CO_2 fixation by NO_2^-. Measurements of CO_2 fixation were performed as described under "Materials and Methods" with the addition that the assays were 33 mM in $NaNO_2$ and contained varying amounts of glucose-6-P. The minus NO_2^- minus glucose-6-P control rate was 72 μmol/mg·Chl·hr.

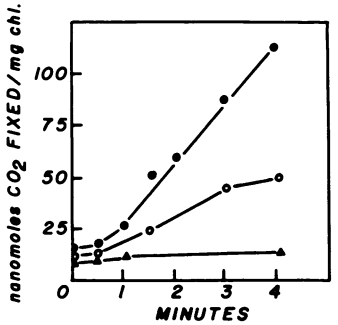

FIG. 3. Enhancement of CO_2 fixation in the dark by glucose-6-P. Measurements of CO_2 fixation were performed as described under "Materials and Methods" except that the dark controls (△) were maintained in darkness from 2 hr prior to the addition of $NaH^{14}CO_3$ and through all measurements of CO_2 fixation; the preilluminated cells (○) were exposed to 10 min of illumination prior to the addition of $NaH^{14}CO_3$ and measurement of CO_2 fixation in the dark; the assays containing glucose-6-P (3 μmol/ml) (●) were incubated with the sugar phosphate for 2 hr in the dark prior to the addition of $NaH^{14}CO_3$ and measurement of CO_2 fixation in the dark.

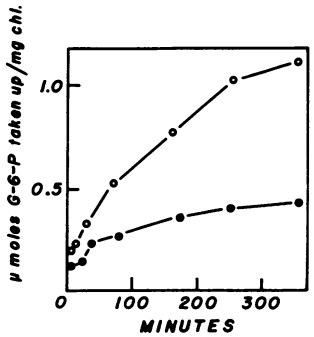

FIG. 4. Uptake of [^{14}C]glucose-6-P in the light and dark by *A. flos-aquae*. Incubation mixture contained cells equivalent to 125 μg Chl and 3.2 μmol glucose-6-P (0.16 μCi/μmol) per ml. Uptake in the light (○); uptake in the dark (●).

probes of the physiology of certain blue-green algae. These results necessitate a direct demonstration of the conditions, rate, and extent to which the metabolite is taken up by the cells.

Uptake of Glucose-6-P. Results of glucose-6-P uptake in either light or dark as a function of time are shown in Figure 4. Uptake is found to be greater in the light than in the dark even though previous studies have indicated that the internal glucose-6-P pool is at its maximum in the light and is regulated such that its turnover is at a minimum in the light (8, 13). Furthermore, the amount of glucose-6-P taken up by these cells is only 10% of the glucose taken up (data not shown). At saturation (350 min), the internal glucose-6-P concentration is calculated to be 1 mM ([uptake/mg Chl] [mg Chl/cell]$^{-1}$ [volume/cell]$^{-1}$). This calculation ignores the endogenous pool and therefore represents a minimum value. The rate of saturation is very slow, indicating that not only the extent, but also the velocity of uptake is limited. This is not unexpected since these cells are primarily autotrophic.

The initial ratio of uptake is saturated in the concentration range one might expect for an enzyme or a specific transport protein (Fig. 5). The $S_{0.5}$ for glucose-6-P uptake in the light was determined to be 6 mM. A true *Km* could not be determined since the uptake kinetics was sigmoidal rather than hyperbolic. Additional evidence for the enzymic nature of uptake was the sensitivity of the rate of uptake to temperature and to pH within physiological ranges. A Q_{10} of 1.82 was calculated between 20 and 30 C and pH optimum of 8 was determined (data not shown).

In order to establish the nature of the molecule taken up when glucose-6-P was supplied, doubly labeled glucose-6-P was synthesized. The results of an uptake experiment using [^{14}C]glucose-6-^{32}P are shown in Figure 6. These data illustrate that the ^{14}C-^{32}P isotopes are taken up in a very similar fashion. When the cells were recovered, washed, and lysed, the major portion of the radioactivity co-chromatographed with glucose-6-P. The percentage distribution of the radioactivity in these recovery experiments was as follows: for ^{14}C, 85% was recovered as glucose-6-P and 15% as either fructose-6-P or 6-P-gluconate, and for ^{32}P, 57% was found as glucose-6-P, 19% as either fructose-6-P or 6-P-gluconate, and 23% as Pi. In addition, the amounts of extracellular acid and alkaline phosphatase were found to be negligible, and the radioactivity recovered from the

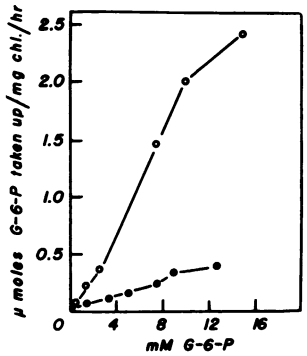

FIG. 5. Saturation curves for the initial rate of glucose-6-P uptake by *A. flos-aquae* in the light and dark. Incubation mixtures contained cells equivalent to 250 μg Chl and varying amounts of glucose-6-P (0.16 μCi/μmol). Uptake in the light (○); uptake in the dark (●).

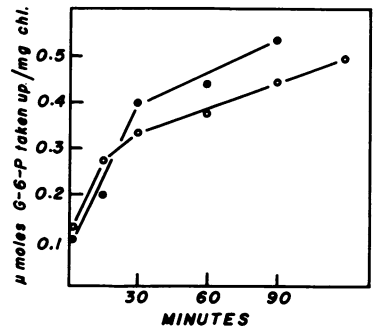

FIG. 6. Assimilation of [^{14}C]glucose-6-P and glucose-6-^{32}P by illuminated cells of *A. flos-aquae*. Uptake of glucose-6-P was performed as described under "Materials and Methods." The 1-ml incubation mixtures contained cells equivalent to 200 μg Chl and 5 μmol glucose-6-P either as [^{14}C]glucose-6-P (0.1 μCi/μmol) (○) or as glucose 6-^{32}P (0.15 μCi/μmol) (●).

medium was associated almost exclusively with glucose-6-P.

The specificity of glucose-6-P uptake was determined by using other sugar phosphates as competitors for glucose-6-P uptake at a limiting glucose-6-P concentration (2 mM). Fructose-1,6-diP (9 mM), gluconate-6-P (9 mM), and ribose-5-P (7 mM) had no effect, while glucose-1-P (9 mM) and fructose-6-P (6.5 mM) inhibited glucose-6-P uptake 50%. The latter two compounds are readily converted to glucose-6-P and could therefore saturate any pools. Whether they might also interact with a specific transport system is unclear. In addition, glucose at 27 mM was without effect on glucose-6-P uptake.

The perturbation of CO_2 fixation by glucose-6-P and the demonstration of its uptake are convincing support for entrance of the glucose-6-P into these algae. Demonstration that glucose-6-P, or any other exogenously supplied metabolite, is regulated by the *in vivo* system in a manner similar to the regulation of *in vivo* pool(s) is required for physiological significance.

Turnover of Glucose-6-P. It was surprising that glucose-6-P uptake was higher in the light than in the dark, since Pelroy and Bassham (13) have reported a lack of turnover of glucose-6-P in the light, and Grossman and McGowan (6) have shown that the enzyme glucose-6-P dehydrogenase can be inhibited by the steady-state concentrations of NADPH and ATP which exist in the light. Our observations were extended to the turnover of glucose-6-P in the light and dark. Utilizing several inhibitors of photosynthesis, it was determined that the turnover of carbon from both glucose and glucose-6-P is much greater in the dark than in the light (Table I). Turnover is defined as $^{14}CO_2$ released from the respective substrate. Presumably this is from carbon 1 of glucose or glucose-6-P because little or no CO_2 is released by these organisms via a tricarboxylic acid cycle (12, 18). Similar experiments [1-^{14}C]glucose-6-P confirmed these results and demonstrated that the glucose-6-P turnover is primarily restricted to the activity of the pentose phosphate pathway. These results supported the previous reports on glucose turnover and the *in vivo* turnover of glucose-6-P (13, 20). In addition, they demonstrate that the uptake of glucose-6-P is not controlled by its oxidation in the cell, but rather is an independently regulated phenomenon.

In a closed system, such as exists in the glucose-6-P turnover experiments, it is difficult to know whether the decreased $^{14}CO_2$ released in the light from glucose-6-P is due to a lack of turnover or to a refixation by photosynthesis. A block in glucose-6-P metabolism in the light has been demonstrated and the kinetics of this blockage investigated (6). Additional evidence that refixation of CO_2 is not a major factor is provided by light saturation experiments (data not shown) in which the inhibition of glucose-6-P turnover is maximal at a light intensity much lower than that which supports appreciable CO_2 fixation. In these experiments, CO_2 fixation was not detectable above the dark rate until the light intensity was greater than 7.5×10^5 ergs cm^{-2} sec^{-1} and showed a linear increase until 1.25×10^6 ergs cm^{-2} sec^{-1}. On the other hand, CO_2 release from glucose-6-P was inhibited 50% at a light intensity of 3.3×10^5 ergs cm^{-2} sec^{-1}. Finally, there is the question of what regulates glucose-6-P turnover. There are several reports (3, 6, 8, 16) which suggest that either ribulose-1,5-diP, ATP, or NADPH, all of which increase in the light, can prevent turnover of glucose-6-P by feedback control to glucose-6-P dehydrogenase. In order to probe this question, glucose-6-P turnover was measured in the presence or absence of the electron transport inhibitor DCMU and the uncoupler FCCP[4] (Table I). In the presence of these inhibitors, glucose-6-P turnover was deregulated in the light. In the presence of FCCP, when NADPH formation should be unimpaired, deregulation occurred, suggesting that either ATP or RuDP is more important than NADPH as an *in vivo* regulator of glucose-6-P turnover.

Table I. The turnover of exogenously supplied ^{14}C-1 glucose and the ^{14}C glucose 6-P by Anabaena flos-aqua

The 1 ml assays contained either 12.5 μmol of radioactive glucose (0.16 μCi/μmol) or 12.5 μmol glucose 6-P (0.16 μCi/μmol).

Conditions	Atmosphere	Inhibitor	CO_2 released from Glucose nmol/mg Chl·hr
light	air	-	8
dark			123
light	air	10μM DCMU	102
dark			112
light	air	100μM FCCP	100
dark			92
			CO_2 released from Glucose 6-P nmol/mg Chl·hr
light	air	-	13
dark			136
light	N_2/CO_2 free air	-	11
dark			124
light	air	10μM DCMU	92
dark			136
light	air	100μM FCCP	148
dark			124

CONCLUSIONS

Our results provide a different approach to the study of why many blue-green algae such as *Anabaena* do not exhibit appreciable growth on either glucose or glucose-6-P in the dark (10). In our experiments, the kinetics of glucose-6-P uptake and turnover is reversed: uptake is greater in the light while turnover is much greater in the dark. These results indicate that uptake is independent of turnover. In addition, uptake is dependent upon photosynthesis while turnover is inhibited by some product(s) of photosynthesis. The results presented here point to the intracellular fluxes of both ATP and reduced pyridine nucleotide as a combined control mechanism preventing heterotrophic growth.

We have determined that, in *Anabaena*, the rate of respiration is 2.4 μmol O_2/mg CHl·hr and 90% of this rate is insensitive to cyanide and azide at 3 mM. Also the inhibition, in *Anabaena*, of O_2 consumption by salicyalhydroxamic acid suggests the presence of an alternate oxidase system, similar to that described for *Vitreoscilla* (21, 22); with a limited capacity for oxidative phosphorylation. Contrary to this suggestion are the data from *Aphanocapsa* 6714 (14) and *Anacystis nidulans* (8) in which the ATP levels in the dark are, after a short lag period, comparable to those in the light.

With respect to reduced pyridine nucleotide reduction, the data are less confused. Ihlenfeldt and Gibson (8) have determined the capacity of *Anacystis* for NADPH oxidation in the dark to be 60 nmol/mg protein·hr and our measurement of O_2-linked NADPH oxidation by *Anabaena* extracts is 30 nmol/mg protein·hr using both spectrophotometric and polarographic methods.

What does this mean with respect to glucose and glucose-6-P uptake and turnover? For *Anabaena*, glucose uptake is not limiting for growth, but for growth on glucose-6-P the dependency on photosynthesis for its uptake makes its uptake limiting. With respect to turnover, glucose and glucose-6-P turnover would appear to be limited in the light by the inhibition by photosynthesis at the glucose-6-P dehydrogenase step and in the dark by insufficient pyridine nucleotide oxidation and therefore insufficient coenzyme for the first step in the pentose phosphate pathway. In fact, the measured rates of NADPH oxidation are in excellent agreement with our dark turnover data.

In summary, some sugar phosphates can be assimilated by blue-green algae and used as probes of autotrophic growth. In the case of glucose-6-P, a branch point intermediate, uptake is stimulated by photosynthesis but turnover is restricted by feedback inhibition from photosynthesis. In the dark, uptake and turnover are limited by the low rate of pyridine nucleotide oxidation (7) exhibited by these cells.

[4] Abbreviation: FCCP: carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone.

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