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Photosynthetic and Dark Carbon Metabolism in Unicellular Blue-Green Algae

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Summary. 1. The kinetics of $^{14}CO_2$ incorporation into cellular intermediates was used to determine the primary pathway of carbon fixation by four genetically diverse unicellular blue-green algae. In each case label was first detected in 3-phosphoglycerate and then in compounds of the reductive pentose cycle.

2. A light to dark transition evoked the same response in all four strains: Immediate cessation of biosynthesis, rapid increase in the concentration of 6-phosphogluconate and changes in the concentrations of sugar mono- and diphosphates. On the other hand, after the first few seconds of dark incubation little or comparatively slow change was noted in the concentrations of 3-phosphoglycerate, phosphoenolpyruvate, citrate, aspartate, and glutamate.

3. For one strain (*Aphanocapsa* 6308) an experiment using both ^{32}P and $^{14}CO_2$ as tracers revealed comparatively rapid turnover of metabolites common to the oxidative pentose cycle during dark incubation. Much slower turnover of labeled carbon was found in other metabolites of glycolysis and the biosynthetic portions of the tricarboxylic acid cycle.

4. During dark incubation of *Aphanocapsa* 6308 the concentration of adenosine triphosphate decreased to approximately 50% of the photosynthetic value within the first 50 sec. In the same period, adenosine diphosphate nearly doubled in concentration. By 4 min after the beginning of the dark period, the steady-state levels of the two adenylates had been restored to photosynthetic levels.

The pathways of photosynthetic carbon fixation and endogenous metabolism in unicellular blue-green algae have not been well characterized. After photosynthesis in the presence of $^{14}CO_2$, the principal

Abbreviations. PGA, 3-phosphoglycerate; G6P, glucose-6-phosphate; RuDP, ribulose-1.5-diphosphate; 6-PGluc, 6-phosphogluconate; FDP, fructose-1.6-diphosphate; SDP, sedoheptulose-1.7-diphosphate; FMP, fructose-6-phosphate; S7P, sedoheptulose-7-phosphate; PEPA, phosphoenolpyruvate; Asp, aspartate; Glut, glutamate; Cit, citrate; HMP, hexose and heptose monophosphates; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

labeled metabolites found include 3-phosphoglycerate (PGA) and various sugar monophosphates and diphosphates which serve as intermediate compounds of the reductive pentose phosphate cycle (Calvin cycle) (Norris *et al.*, 1955). Photosynthetic carbon fixation has been presumed to be by means of the reductive pentose phosphate cycle in blue-green algae (Holm-Hansen, 1968; Evans and Whatley, 1970), although the kinetic analyses of $^{14}CO_2$ incorporation into metabolic pools necessary for this conclusion have not been reported to date. That the Calvin cycle is the means of CO_2 reduction in *Anacystis nidulans* is supported by the finding that the distribution of ^{14}C within glucose molecules after a short period of photosynthesis in these organisms is similar to that found in glucose from the green alga *Chlorella pyrenoidosa* (Kindel and Gibbs, 1963).

It is known that the tricarboxylic acid cycle (TCA cycle) is incomplete in several species of unicellular and filamentous blue-green algae (Smith *et al.*, 1967; Hoare *et al.*, 1967; Pearce and Carr, 1969; Van Baalen *et al.*, 1971). The fact that incorporation of ^{14}C from ^{14}C-labeled acetate is limited to glutamate and the glutamate family of amino acids, the aliphatic amino acids leucine and isoleucine, and lipids indicates a block in the oxidation of alpha ketoglutarate to succinate. This is corroborated by the lack of alpha keto glutarate dehydrogenase activity in cell extracts of these organisms. Uptake of acetate is strictly dependent on light and CO_2. Thus, cells are incapable of oxidizing acetate to obtain energy as ATP for the conversion of acetate to cell material.

We shall describe here the primary pathway of CO_2 fixation in four strains of unicellular blue-green algae, selected for maximal divergence with respect to the mean base composition of their DNA (Stanier *et al.*, 1971). The methodology was basically the same as used to establish the pathway of carbon dioxide fixation in green algae (Benson *et al.*, 1950; Bassham and Kirk, 1960; Pedersen *et al.*, 1966), and involved measurement of the kinetics of ^{14}C appearance in the intermediary pools of the organisms studied. The second objective of this study was to determine the changes in metabolite concentration in cells after the transition from light to dark. From analysis of these changes it was hoped that insight into the pathway(s) of endogenous metabolism might be gained.

Materials and Methods

Organisms

Synechococcus 6301 *(Anacystis nidulans)*, *Synechococcus* 6307 *(Coccochloris peniocystis)*, *Aphanocapsa* 6308 *(Gleocapsa alpicola)*, and *Aphanocapsa* 6714, all part of the Berkeley culture collection, were used in this study. The nomenclature of these strains is that proposed by Stanier *et al.* (1971); previous designations (if any) are shown in parentheses.

Media and Culture Conditions

Cells were grown photoautotrophically in an inorganic liquid medium, BG-11, described elsewhere (Stanier *et al.*, 1971).

^{14}C Tracer Experiments

Cells grown on BG-11 were harvested at room temperature by centrifugation at 17,000 g for 20 min and resuspended in BG-11 at a concentration of 1% w/v. The algal suspension was then placed in the steady-state apparatus previously described (Bassham and Kirk, 1964). The chamber containing the algae is a transparent vessel, equipped with a water jacket, inlet and outlet valves for gas, a sampling valve, pH electrodes, a densitometer and automatic pH control and was maintained at constant temperature of 25° C. The light sources were two General Electric photo-DXB incandescent bulbs. The light was first passed through infrared filters to minimize heating. The gas phase was air, enriched with 0.5% CO_2 and circulated by a small diaphragm pump. The levels of CO_2, O_2, and ^{14}C in the gas phase were all monitored automatically. The system was closed and a bypass containing a loop of $^{14}CO_2$ was introduced by a four-way stopcock.

When the cells had achieved the maximum rate of photosynthesis, monitored by O_2 evolution and CO_2 consumption, the stopcock separating $^{14}CO_2$ from the system was opened, allowing the isotope to be circulated through the algae by bubbling. The specific activity of CO_2 after addition of the tracer was 20.8 C/mole for *Synechococcus* 6301 and *Aphanocapsa* 6308 and 22.1 C/mole for *Synechococcus* 6308 and *Aphanocapsa* 6714. After 10 min of photosynthesis, the lights were turned off and the remainder of the experiment carried out in the dark. Sampling during the course of the experiments was done automatically, samples of approximately 1 ml being introduced into weighed test tubes containing 4 ml of methanol. These tubes were again weighed to determined sample size.

Radioactive compounds formed by the algae were analyzed by two-dimensional paper chromatography and radioautography as previously described (Pedersen *et al.*, 1966). Chromatograms were developed in two directions using Whatman No. 1 chromatographic paper. The solvent system was phenol-water-glacial aretic acid-ethylenediamine tetraacetic acid (1 M) (840:160:10:1 v/v) in the first direction. The second solvent was made up of equal volumes of *n*-butanol-water (370:25 v/v) and propionic acid-water (180:220 v/v) as described (Benson *et al.*, 1950). Chromatograms were developed 24 h in each direction for the resolution of amino acids, phosphoglyceric acid (PGA), phosphopyruvate (PEPA) and citrate (Cit). For the analysis of mixtures of glucose-6-phosphate (G6P), sedoheptulose-7-phosphate (S7P), fructose-6-phosphate (FMP), 6-phosphogluconate (6-PGluc), fructose-1.6-diphosphate (FDP), sedoheptulose-1.7-diphosphate (SDP), and ribulose-1.5-diphosphate (RuDP), the chromatograms were developed 48 h in each direction. In these experiments the sugar phosphates and diphosphates were then dephosphorylated by treatment with phosphatase after elution of the radioactive mixtures with distilled water. The radioactive sugars were then resolved by rechromatography, using the same solvent systems.

^{14}C and ^{32}P Tracer Experiments

For certain experiments with *Aphanocapsa* 6308, isotopes of both carbon and phosphorus were used to measure the synthesis of radioactive material. The procedure of radioautography was modified as described previously (Pedersen *et al.* 1966) to remove inorganic ^{32}P to prevent complete development of X-ray films. In

these experiments the final specific activity of $^{14}CO_2$ was 21.6 C/mole. Approximately 5 mC of ^{32}P was used to label phosphorus-containing compounds.

For experiments with either one or two radioactive tracers, areas of paper containing radioactive material, determined by radioautography, were cut out of the chromatograms and counted by means of the "automatic spot-counter" developed by Moses *et al.* (1963), as modified to permit simultaneous measurement of ^{14}C and ^{32}P (Krause *et al.*, 1968).

Results

Light Phase

Fig. 1—2 show the kinetics of $^{14}CO_2$ incorporation into the metabolic pool of *Aphanocapsa* 6308. As expected for an organism using the Calvin cycle, PGA was the first compound to be labeled (Fig. 1). Within 30 sec to 1 min, label was detectable in RuDP, G6P, FMP, and S7P. Thus labeled carbon is first incorporated into PGA and then appears to be flowing through the intermediates of the Calvin cycle. Also labeled in the first 60 sec are PEPA and Asp (Fig. 2). After about 3 min, ^{14}C appears in glutamate.

Fig. 3 shows the incorporation of labeled carbon into insoluble material in the four species studied. Subsequent treatment of this material with amylase solubilized nearly all of the radioactivity which was tentatively identified as a mixture of glucose, di- and tri-saccharides and short chain polysaccharides (data not shown). Acid hydrolysis of amylase-solubilized material yielded a single compound identified by chromatography as

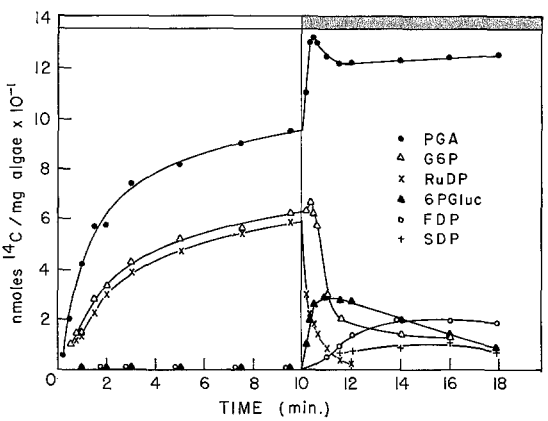

Fig. 1. ^{14}C-labeled intermediates in *Aphanocapsa* 6308 during photosynthesis and a subsequent dark period: labeling of PGA, G6P, RuDP, 6-PGluc, FDP, and SDP. The experiment was carried out in the steady-state apparatus by the introduction of $^{14}CO_2$ at 0 time. Carbon concentrations are expressed in terms of wet weight of whole cells. Open bar (0—10 min) indicates photosynthesis; shaded by (10—20 min) indicates dark incubation

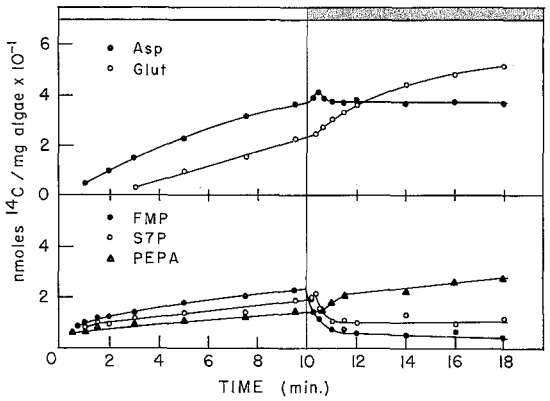

Fig. 2. Concentrations of ^{14}C-labeled intermediates in *Aphanocapsa* 6308 during photosynthesis and dark incubation: Labeling of Asp, Glut, FMP, S7P, and PEPA. Experimental conditions and specific activities of metabolites the same as Fig. 1

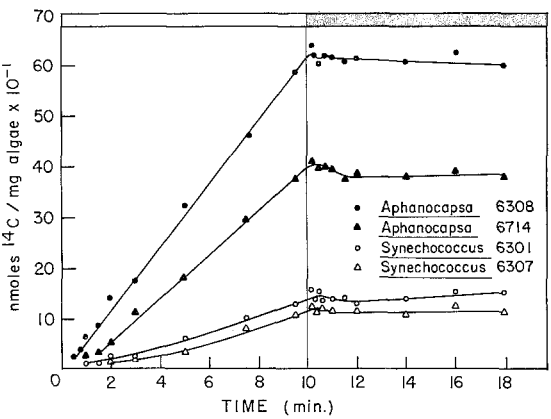

Fig. 3. Incorporation of ^{14}C into polyglucose by four strains of unicellular blue-green algae in the light and in the dark. Experimental conditions and specific activities same as for Fig. 1

glucose (Methods). Thus we conclude that the only polymer synthesized in significant amounts during these experiments was a poly-glucose, probably glycogen.

The results of carbon dioxide incorporation experiments for three unicellular blue-green algae are shown in Fig. 4. In these experiments monophosphate and diphosphate sugars were not separated and analyzed individually. However, the kinetics of CO_2 fixation into intermediary

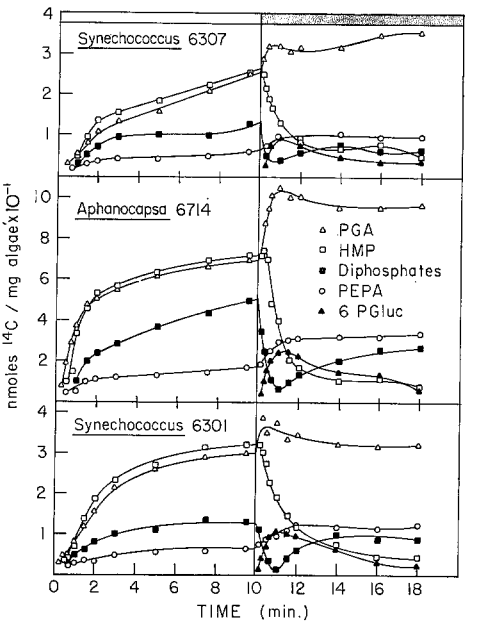

Fig. 4. Concentrations of ^{14}C-labeled intermediates in 3 strains of unicellular blue-green algae. Experimental conditions and specific activities of metabolites same as Fig. 1

metabolites was essentially the same as for *Aphanocapsa* 6308, with labeling of PGA first, followed by sugar monophosphates, sugar diphosphates, and PEPA. Thus, the kinetics of carbon-14 assimilation suggests the operation of the Calvin cycle.

Dark Phase

Figs. 1—3 also show the changes that occur in metabolite pools resulting from a light-to-dark transition in *Aphanocapsa* 6308. ^{14}C incorporation into glycogen stops almost instantaneously after the light is turned off (Fig. 3). Also a nearly instantaneous surge of carbon appears in PGA, concomitant with the loss of labeled carbon from RuDP (Fig. 1). After a 30 sec delay, a loss of label also occurs in the sugar phosphates, G6P, FMP, and S7P (Figs. 1 and 2). Within the first 15 sec of the dark phase, ^{14}C begins to appear in 6-PGluc and a little later (30 sec) fructose-1,6-diphosphate (FDP) and sedoheptulose-1,7-diphosphate (SDP) become labeled. The incorporation of ^{14}C into Asp ceases within 30 sec into the dark phase. On the other hand, there is a slow accumulation of labeled carbon in Glut throughout the 8-min dark phase.

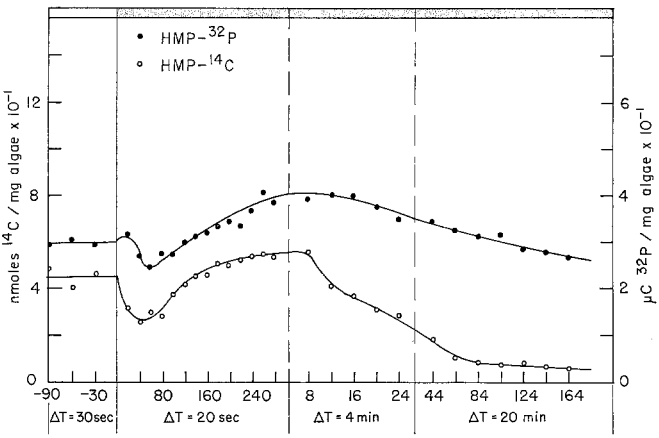

Fig. 5. ^{32}P and ^{14}C in HMP during incubation of *Aphanocapsa* 6308. $^{14}CO_2$ and ^{32}P were introduced into the steady-state apparatus containing an algal suspension at minus 20 min. Samples were taken to establish the photosynthetic pool size (open bar) and then the lights were turned off at 0 time (shaded bar). Specific activities of intermediates are expressed in terms of wet weight whole cells

The changes during dark periods with the three other unicellular blue-green algae (Fig. 4) were similar to those observed for *Aphanocapsa* 6308. ^{14}C was quickly lost from sugar mono- and diphosphates, increased in PGA and PEPA, and appeared for the first time in 6-PGluc. As stated above, the mono- and diphosphate sugars were not separated for further analysis. Thus the dark periods for all four of the unicellular blue-green algae examined were similar, suggesting that changes in metabolism that occurred were the same in each case.

Double Labeling Experiments

The use of both ^{14}C and ^{32}P allowed the detection of turnover in the metabolite pools of *Aphanocapsa* 6308. In the dark, ^{14}C was diluted out of monophosphates (G6P + S7P) with a half time of approximately 20 min (Fig. 5). Presumably this dilution is due to the flow of ^{14}C into these pools from unlabeled endogenous carbohydrate reserves (glycogen). The two sugars were not separated in this experiment, since the phosphorus moiety must be hydrolyzed before chromatographic separation (Methods). Thus, the sum of radioactivity in both sugars is presented in Fig. 5. However, as shown in Figs. 1 and 2, approximately 67% of the radioactivity was present in G6P at the end of the light period; thus the curves of Fig. 5 are probably representative of this compound.

In contrast to the results for ^{14}C, the radioactivity in ^{32}P decreased only slightly during dark incubation, suggesting that the pools of G6P

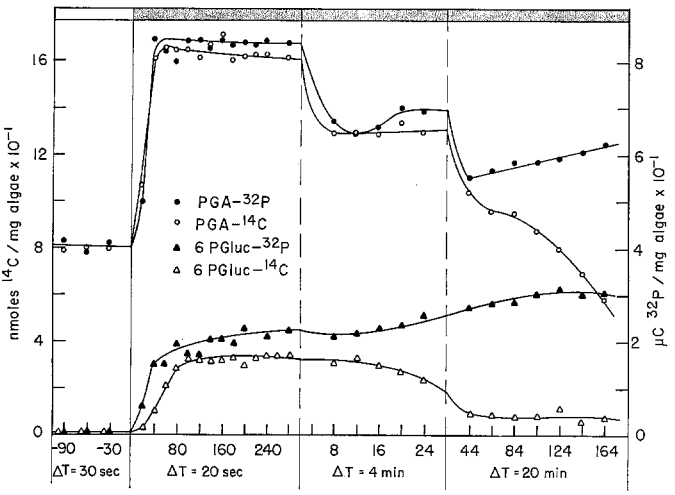

Fig. 6. Concentrations of ^{32}P and ^{14}C in PGA and 6-PGluc in *Aphanocapsa* 6308 during dark incubation. Experimental conditions and specific activities of metabolites the same as for Fig. 5

and S7P did not substantially change in size. ^{32}P incorporation into metabolites is of course the same, whether via photosynthetic phosphorylation, oxidative phosphorylation, or phosphorolysis of glycogen.

Fig. 6 shows ^{14}C and ^{32}P labeling of PGA and 6-PGluc. As was the case for the sugar monophosphates, ^{14}C was diluted out of 6-PGluc with a half time of between 16 and 20 min, while the ^{32}P concentration remained essentially constant. The curves for PGA are more complex. Both ^{32}P and ^{14}C change together for the first 44 min. Between 44 and 164 min ^{14}C was diluted out of the PGA pool (half time of about 60 min), while the ^{32}P label (total pool indicator) increased slightly. Accordingly, carbon was turning over in both PGA and 6-PGluc during dark incubation. However, the rate of turnover in the PGA pool was only $^1/_3$—$^1/_4$ the rate observed for the sugar monophosphates and 6-PGluc. Thus, the data suggest that dark incubated cells of *Aphanocapsa* 6308 metabolize glucose much more actively than PGA.

The concentrations of glutamate, aspartate, and citrate during dark incubation are shown in Fig. 7. As can be seen, only the glutamate pool increased significantly in size during this period. The rise in radioactivity began at about 8 min and ceased near the end of the experiment between 124 and 164 min. On the other hand, the concentrations of citrate and aspartate were nearly constant throughout the dark period. Thus ^{14}C began to flow more rapidly into glutamate at about the same time the PGA pool began to lose ^{14}C more rapidly.

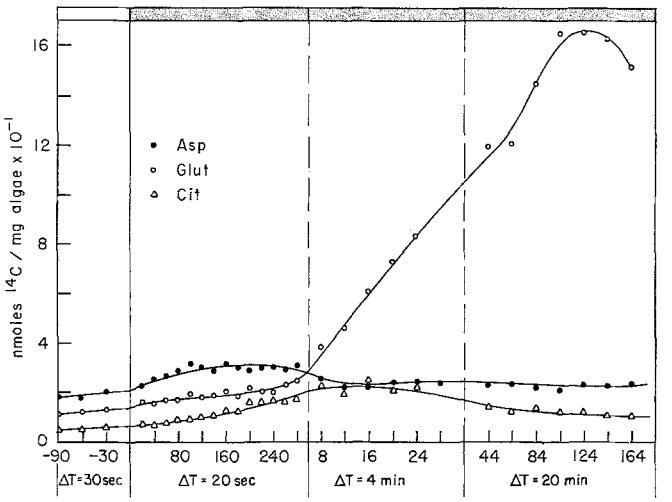

Fig. 7. Concentrations of ^{14}C in Asp, Glut, and Cit during dark incubation of *Aphanocapsa* 6308. Experimental conditions and specific activities of metabolites same as for Fig. 5

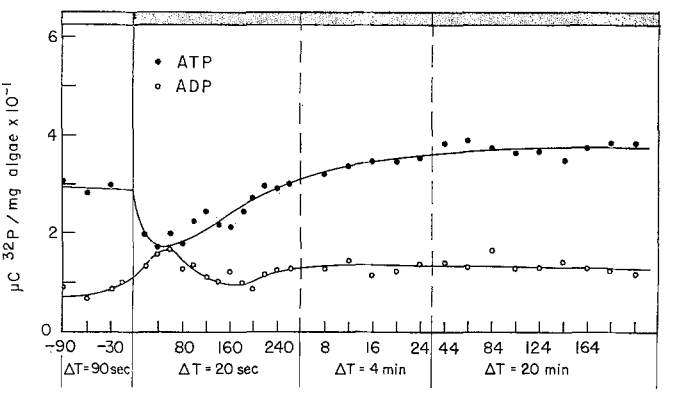

Fig. 8. Concentrations of ^{14}C and ^{32}P in ATP and ADP during dark incubation of *Aphanocapsa* 6308. Experimental conditions and specific activities of metabolites same as for Fig. 5

ATP and ADP in the Dark Period

The concentration of ^{32}P in ATP decreased abruptly on entering the dark phase (Fig. 8), while ADP increased in concentration. However, at approximately 1 min after the light source was removed, radioactivity in ATP began increasing, equalling the photosynthetic level between 4 and 8

min of dark incubation. Thus *Aphanocapsa* 6308 possessed the means for regenerating ATP depleted in the light-dark transition, and for maintaining ATP at concentrations comparable to those of photosynthetic metabolism.

Discussion

The kinetics of ^{14}C incorporation into metabolites during photosynthesis are strong evidence for the operation of the reductive pentose cycle (Calvin cycle) as the principal means of carbon reduction in the four unicellular blue-green algae used in this study. The genetic diversity of these strains was large, spanning the entire range of DNA base composition found in the *Chroococcales*: *Aphanocapsa* 6308, 34.7 moles % GC; *Aphanocapsa* 6714, 47.4 moles % GC; *Synechococcus* 6301, 55.1 moles % GC; *Synechococcus* 6307, 69.7 moles % GC (Stanier *et al.*, 1971). Accordingly, the Calvin cycle appears to be the characteristic, if not the sole path of CO_2 fixation in this group of microorganisms.

A light-dark transition in *Aphanocapsa* 6308 produced a rapid cessation of biosynthesis, and of Calvin cycle activity, together with activation of a pathway that yielded increased concentrations of 6-PGluc, DHAP, FDP and SDP.

The appearance of 6-PGluc apparently results from glucose-6-phosphate oxidation and suggests that G6P dehydrogenase increases in activity as soon as the dark period begins. Moreover, the loss of ^{14}C label from G6P with the appearance of radioactivity in 6-PGluc, the slower dilution of label from the latter compound, followed by the appearance of ^{14}C in FDP, suggests these compounds are formed sequentially during the endogenous metabolism of glucose by *Aphanocapsa* 6308.

The increased concentration of 6-PGluc, together with increased levels of FDP and SDP, suggest operation of the oxidative pentose phosphate pathway. FDP and SDP must also be produced in the light during operation of the Calvin cycle, but apparently the steady-state concentrations of these compounds were too low to be detected in this experiment.

FDP accumulation in the dark period might be interpreted as an indication of glycolytic activity. However, as shown elsewhere (Pelroy *et al.*, 1972), cell-free extracts of this organism are devoid of phosphofructokinase (PFK) activity. Thus it is more likely that FDP results from the action of fructose diphosphate aldolase on triose phosphate formed by the oxidative pentose phosphate cycle.

It is of interest that the levels of glucose-6-phosphate and 6-phosphogluconate dehydrogenase are comparatively high in cell free extracts of these organisms (Pelroy *et al.*, 1972). Thus the enzymatic machinery for the initial steps of the oxidative pentose pathway are present. Most of the remaining enzymes which are required for the oxidative pentose pathway also operate in the Calvin cycle. On the other hand, enzyme activities

specific to the Entner-Doudoroff pathway, and, as indicated above, phosphofructokinase activity, are not detectable.

The rapid increase of radioactivity in PGA (Fig. 1) in the first few seconds of the dark phase is presumably due to the carboxylation reaction continuing in the dark (using up the large pool of RuDP) while the reduction of PGA to triose phosphate ceases due to exhaustion of the supply of NADPH from the light reactions. The constancy of the PGA pool after the first minute of dark incubation suggests this compound is metabolized much more slowly than the sugar phosphates and 6-PGluc. This agrees with the observed accumulation of PEPA in the dark phase. The higher levels of both PGA and PEPA in the dark, after changes, suggest that pyruvic kinase remains rather inactive during dark incubation. The slow metabolism of PGA and the accumulation of PEPA in the dark are not observed in *Chlorella pyrenoidosa* (Kanazawa *et al.*, 1972) where there is a fairly rapid metabolism of PGA to TCA cycle intermediates, and a rapid onset of oxidative phosphorylation in the dark. In this eucaryotic green alga, the TCA cycle in the mitochondria is complete.

The pool changes in the dark in the other three strains examined were essentially the same as in *Aphanocapsa* 6308. The concentration of ^{14}C in diphosphates (mainly RuDP) and monophosphates (mainly G6P) decreased abruptly, while label was accumulating in 6-PGluc. These data suggest that the same pathway is activated after a light-dark transition in all four unicellular blue-green algae.

In the double labeling experiments with *Aphanocapsa* 6308, ^{14}C was diluted out of the sugar phosphates G6P and S7P much faster than ^{32}P, indicating the pool sizes for these compounds were relatively constant in the light and in the dark and that carbon tracer was being replaced by unlabeled material from an endogenous source (probably glycogen). The turnover of carbon in PGA was much slower, at least by a factor of 4. Accordingly, carbon was passing through the sugar phosphates and 6-PGluc much faster than through PGA during the dark period. Thus, the major metabolic activity during the dark period affected metabolites associated with the oxidative pentose pathway, not with glycolysis or the Entner-Doudoroff hexose monophosphate pathway.

For one of the strains, *Aphanocapsa* 6714, the evidence implicating the oxidative pentose pathway is more direct. This organism is capable of heterotrophic growth in the dark using glucose as the carbon source (Rippka, 1972), a property very rare in the unicellular blue-green algae. Moreover, experiments using differentially labeled glucose (Pelroy *et al.*, 1972) have provided strong evidence for the oxidative pentose phosphate pathway as the means of glucose catabolism. It is of interest that this pathway has also been demonstrated in the filamentous heterotroph, *Tolypothrix tenuis* (Cheung and Gibbs, 1965) and evidence has been

presented implicating this pathway in the filamentous photolithotroph, *Anabaena variabilis* (Pearce and Carr, 1969), and several chemolithotrophic thiobacilli (Matin and Rittenberg, 1971). The heterotrophic thiobacilli, on the other hand, dissimilate glucose through an inducible Entner-Doudoroff pathway (Matin and Rittenberg, 1970; Tabita and Lundgren, 1971).

It is clear that the oxidative pentose pathway would be well suited for maintaining energy requirements during endogenous metabolism by organisms that are strictly or predominantly lithotrophic. A high ratio of reduced pyridine nucleotides to substrate (6 reduced NADP in a cyclic pathway) would yield a large amount of potential energy. It would not be necessary to "prime" glucose with ATP (as in glycolysis) if glycogen were a reserve material of the cell, as is the case for both the unicellular blue-green algae and the thiobacilli. In addition, the oxidative pentose cycle would not produce toxic pH changes due to the formation of organic acids.

Regardless of which particular pathway is being used for catabolism, it is clear that *Aphanocapsa* 6308 does possess the means for replenishing and maintaining ATP during the dark period. However, the use of the oxidative pentose cycle would almost certainly require respiratory rather than fermentative capacity. It has been known for some time (Biggins, 1969) that concentrations of ATP and reduced NADP in *Synechococcus* 6301 (*Anacystis nidulans*) were lower under anaerobic conditions, and that the low uptake by this organism could be stimulated by the uncoupler of oxidative phosphorylation, dinitrophenol.

The means by which endogenous metabolism is controlled is of interest. The rapid rise in 6-PGluc concentration early in the dark period suggests that glucose-6-phosphate or 6-phosphogluconate dehydrogenase is under metabolic control. Secondly, the very slow rate of carbon turnover in PGA, PEPA, Asp, and Glut relative to that observed in the sugar phosphates and 6-PGluc suggests controls that operate to prevent the late reactions of glycolysis (possibly at the conversion of PEPA to pyruvate), thus effectively limiting the synthesis of carbon skeletons required for biosynthesis.

Many of the pool changes during a light to dark transition in the unicellular blue-green algae used in this study resemble those in *Chlorella pyrenoidosa* (Pedersen *et al.*, 1966; Kanazawa *et al.*, 1972). Especially interesting is the rapid rise in 6-PGluc concentration in the chloroplasts of this eukaryotic green alga. It may be that the chloroplasts and the unicellular blue-green algae contain many regulatory features in common, possibly indicating controls that existed in a common ancestor.

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