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Kinetics of Light-Dark CO_2 Fixation and Glucose Assimilation by *Aphanocapsa* 6714

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Cells of *Aphanocapsa* 6714 were subjected to alternating light-dark periods (flashing-light experiments). The corresponding activation (in the light) and inactivation (in the dark) of the reductive pentose cycle was measured, in vivo, from initial rates of $^{14}CO_2$ incorporation and also by changes in the total concentration of ^{14}C and ^{32}P in soluble metabolites. Two principle sites of metabolic regulation were detected: (i) CO_2 fixation was inactivated 15 to 20 s after removal of the light source, but reactivated rapidly on reentering the light; (ii) hydrolysis of fructose-1,6-diphosphate (FDP) and sedoheptulose-1,7-diphosphate (SDP) by their respective phosphatase(s) (FDP + SDPase) was rapidly inhibited in the dark but only slowly reactivated in the light. The time required for reactivation of FDP + SDPase, in the light, was on the order of 20 to 30 s. As a consequence of the timing of these inactivation-reactivation reactions, newly fixed CO_2 accumulated in the FDP and SDP pools during the flashing-light experiments. Changes in the concentrations of the adenylate pools (mainly in the levels of adenosine 5'-triphosphate and adenosine diphosphate) were fast in comparison to the inactivation-reactivation reactions in the reductive pentose cycle. Thus, these regulatory effects may not be under the control of the adenylates in this organism. The activation of CO_2 fixation in the light is at least in part due to activation of phosphoribulokinase, which is required for formation of ribulose-1,5-diphosphate, the carboxylation substrate. Phosphoribulokinase activity in crude extracts was found to be dependent on the presence of strong reducing agents such as dithiothreitol, but not significantly dependent on adenylate levels, although adenosine 5'-triphosphate is a substrate.

The reductive pentose cycle is the sole means of photoautotrophic CO_2 fixation by the unicellular blue-green alga *Aphanocapsa* 6714 (16, 19). When cells are placed in the dark, CO_2 fixation ceases and oxidative metabolism of glucose-6-phosphate (G6P), via the oxidative pentose cycle, begins immediately. G6P oxidation in the light is prevented by high ratios of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to NADP (18) and also by inhibition by ribulose-1,5-diphosphate (RuDP) (19).

In the present study, our objective was to identify the principle sites in the reductive pentose cycle that are inactivated when cells change from photosynthetic to respiratory (dark) metabolism. In a series of flashing-light experiments, the initial rates of $^{14}CO_2$ fixation and changes in levels of the metabolite pools at the reductive pentose cycle were measured. In these experiments, inorganic phosphate labeled with ^{32}P was administered to provide a second tracer for sugar phosphates (and related phosphorylated metabolites) and also to permit observation of changes in the adenylate pools.

MATERIALS AND METHODS

Growth. *Aphanocapsa* 6714 was grown on a mineral base medium, BG-11, supplemented as described previously (17). Cells were grown from small inocula in the presence of approximately 20 μCi of carrier-free ^{32}P per ml (17), ensuring complete saturation of the tracer in phosphorous-containing metabolites. When cells had grown to an optical density of 120 optical density units, they were harvested (500 ml per side-arm flask), washed in phosphate-free BG-11 medium, and resuspended in 50 ml of BG-11 medium minus phosphate. Sodium bicarbonate solution was added to make the cell suspension about 30 mM, and then the cells were placed in the steady-state machine, as previously described (15, 17).

Double-labeling experiments: ^{14}C and ^{32}P. Two types of labeling experiments were conducted. In initial-velocity experiments, the cells were prelabeled with ^{32}P, and the ^{14}C label was introduced to initiate the experiment. In steady-state experi-

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ments, the cells were also prelabeled with ^{32}P, but the ^{14}C label (as $^{14}CO_2$) was added 30 min before beginning the experiment. Since ^{14}C enters intermediary metabolism much more quickly than ^{32}P, it is possible to achieve steady-state concentrations in this comparatively short time.

In initial-velocity experiments (i.e., where the rate of ^{14}C assimilation was measured) the specific activities for carbon were: $^{14}CO_2$, 10.02 μCi/μmol; [U-^{14}C]glucose, 8.4 μCi/μmol. The concentration of $^{12}CO_2$ plus $^{14}CO_2$ in the gas phase was 0.5%, vol/vol. For the adenosine nucleotides, the value of ^{32}P was divided by the number of phosphorous atoms per molecule—i.e., to give nanocuries or microcuries per microgram-atom of phosphorous—a measure of concentration (14, 15). In steady-state experiments, in which cells were prelabeled with both phosphorous and carbon tracers, the specific activities of $^{14}CO_2$ and ^{32}P were: 22 μCi/μmol and approximately 20 μCi/μmol, respectively. The amount of labeled material is expressed as concentration of label per milligram (dry weight) of algae in all figures.

To begin an initial-velocity flashing-light experiment (see below), $^{14}CO_2$ or [U-^{14}C]glucose was introduced into the steady-state system. Cells initially contained no ^{14}C. Phosphate groups in metabolites were already fully labeled with ^{32}P. In time, $^{14}C/^{32}P$ ratios approached constant values that are characteristic for a given metabolite. Thus, $^{14}C/^{32}P$ ratios were a measure of specific activity of a given metabolite.

On an equivalent molar basis, 3-phosphoglycerate (PGA) and fructose-1,6-diphosphate (FDP) contain a ratio of phosphorous to carbon of 1:3, which is twice that of the hexose monophosphate G6P. Thus, at any given time between zero time and when saturating (steady-state) concentrations of ^{14}C are reached, the $^{14}C/^{32}P$ ratios of these two classes of metabolites will be related by a constant factor. For comparing compounds on an equivalent basis, we arbitrarily chose to make all comparisons relative to hexose monophosphates. Thus, $^{14}C/^{32}P$ ratios for PGA and FDP were multiplied by a factor of 2 to make them equivalent to $^{14}C/^{32}P$ ratios for G6P when comparisons of specific activity in the different pools were to be made.

In our initial-velocity experiments, we were forced to make assumptions about the FDP + sedoheptulose-1,7-diphosphate (SDP) and G6P + sedoheptulose-7-phosphate (S7P) pools. The diphosphates, as well as the monophosphates, tend to move together in the solvent systems used for paper chromatography (15). These compounds can be separated by secondary chromatography; however, this requires hydrolysis of the phosphate groups followed by rechromatography and radioautography of ^{14}C remaining in the dephosphorylated sugars. This would have been difficult or impossible in the early stages of the initial-velocity experiments because levels of ^{14}C were quite low. However, over the course of many experiments, we have noted that concentrations of FDP were always two to four times the level of SDP, whereas the concentrations of G6P were three to nine times the concentration of S7P. Thus, only a small error was introduced into specific activity calculations by assuming all phosphorous in FDP + SDP was in FDP (3 carbons per phosphorous) and all phosphorous in G6P + S7P was in G6P (6 carbons per phosphorous).

Flashing-light experiments. To begin a flashing-light experiment (initial velocity) $^{14}CO_2$ or [U-^{14}C]glucose was added to cells prelabeled with ^{32}P, as described above. At the beginning of the experiment, cells had been in continuous light long enough for steady rates of photosynthesis to be established. As the ^{14}C tracer was added to the system, the lights were turned off for a half-cycle of 45 s and then turned on again for the second half-cycle of 45 s. This schedule was followed through several cycles, with samples taken every 15 s. Samples were immediately killed in 80% methanol, and the radioactivity in soluble metabolites was determined by two-dimensional paper chromatography followed by radioautography, as previously described (15). Radioactivity in the separated metabolites on the chromatograms was determined by gas flow spectrometry on an automated counter, described in detail elsewhere (14, 15). In the steady-state experiment, where both carbon and phosphorous tracers in the reductive pentose cycle were prelabeled to steady-state concentrations, the schedule consisted of 40-s light-dark half-cycles, with samples taken every 10 s.

Assay of PRK. Cells were stored frozen in a paste at minus 20°C. They were thawed and resuspended in a standard buffer consisting of: 10 mM triethanolamine (pH 7.5), 1.0 mM NaCl, and 0.2 mM dithiothreitol (DTT), or 0.2 mM β-mercaptoethanol and 10 mM $MgCl_2$. Each subsequent step was done in this buffer. The cells were broken with a French pressure cell using 20,000 lb/in^2; usually the cells had to be passed several times through the cell to obtain maximum breakage. Broken cells were centrifuged for 15 min at 36,000 $\times$ g, and the supernatant fraction was saved and centrifuged a second time, 60 min at 36,000 $\times$ g. The clear, dark blue-green supernatant fraction was used for the assay of phosphoribulokinase (PRK) in cell-free extract. The reaction mixture for the enzyme consisted of the following reagents in 1 ml: $MgCl_2$, 10 mM; tris(hydroxymethyl)aminomethane (pH 7.5), 20 mM; adenosine 5'-triphosphate (ATP), 0.5 mM; adenosine diphosphate (ADP), 0 or 20 mM; ribulose 5-phosphate (Ru5P), 0 or 1.2 mM; DTT or β-mercaptoethanol, 0 or 1.0 mM; NADPH, 0 or 1.0 mM; NADH, 0 or 1.0 mM; purified spinach RuDP carboxylase (PRK-free), 5 U; and $NaH^{14}CO_3$, 20 mM (5.0 μCi/μmol). The reaction was started with the addition of enough crude extract to catalyze the fixation of about 0.1 μmol of $^{14}CO_2$/min in the coupled enzyme reaction. The crude enzyme preparation was kept unfrozen at 4°C until used.

The reactions were carried out in 10-ml vials, 1 ml per vial. The enzymes were denatured by the addition of 250 μl of 3 N trifluoracetic acid, and the contents of each vial were evaporated to dryness under a nitrogen stream. Five-tenths milliliter of water was added to each of the dried vials and the

contents were resuspended; then 6.5 ml of Aquasol (New England Nuclear Corp.) was added. The vials were capped and shaken to form a light blue-green solution in which radioactivity in PGA was determined (as a measure of PRK activity).

RESULTS

Initial-velocity experiments: ^{14}C and ^{33}P. When $^{14}CO_2$ fixation rates were compared with ADP and ATP levels during alternating 45-s periods of light and darkness (Fig. 1), no clear immediate dependence of CO_2 fixation on the ratio of ATP/ADP was seen. Fixation of CO_2 began immediately and was approximately linear during the light periods, although there was a slow rate of uptake during the first cycle (probably owing to time required for equilibration of already present CO_2 with $^{14}CO_2$ tracer). In contrast, ATP/ADP ratios were low during the dark periods (e.g., about 1:1) and rose rapidly in the initial seconds of each light period (about 5:1 within 15 s in the light).

Except for the initial dark period, CO_2 was fixed more rapidly during the first 15 s of each dark period than during the preceding light period. Over the course of the entire experiment, CO_2 fixation in the initial 15 s of the dark periods accounted for nearly one-third of all CO_2 fixed and occurred at a rate at least 1.5 times the light fixation rate. Perhaps this accelerated dark fixation rate (just after a light period) is similar to the burst of CO_2 fixation in the dark after photosynthesis reported by Emerson and Lewis (6), in studies with *Chorella*.

Figures 2 through 5 show the fate of CO_2 fixed during the experiment of Fig. 1. Each figure is in two parts: top panels show ratios of $^{14}C/^{32}P$, a measure of specific activity (see Materials and Methods); the bottom panels show the content of ^{14}C in the metabolite pools of the reductive pentose cycle.

The concentration of ^{14}C in PGA increased and decreased in a cyclic manner (bottom, Fig. 2). Noteworthy were the rapid increases of ^{14}C

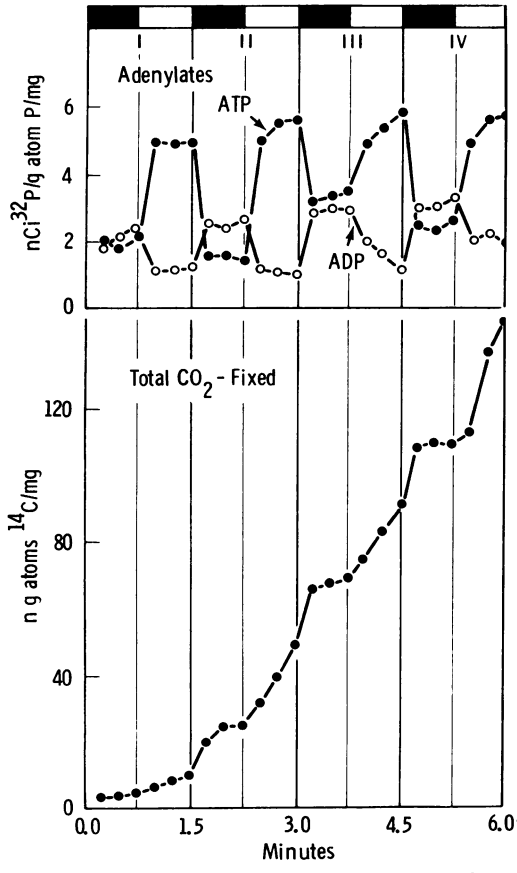

FIG. 1. *Adenylate concentrations (top) and net CO_2 fixation (bottom) in a flashing-light experiment. Samples were taken every 0.25 min (15 s); the light and dark periods were 0.75 min (45 s) each.*

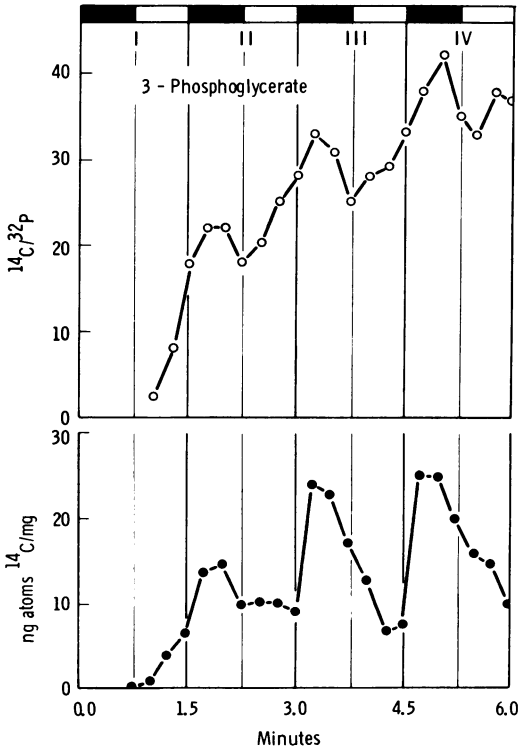

FIG. 2. *$^{14}C/^{32}P$ ratios (top) and concentration of ^{14}C in PGA (bottom) during the flashing-light experiment of Fig. 1.*

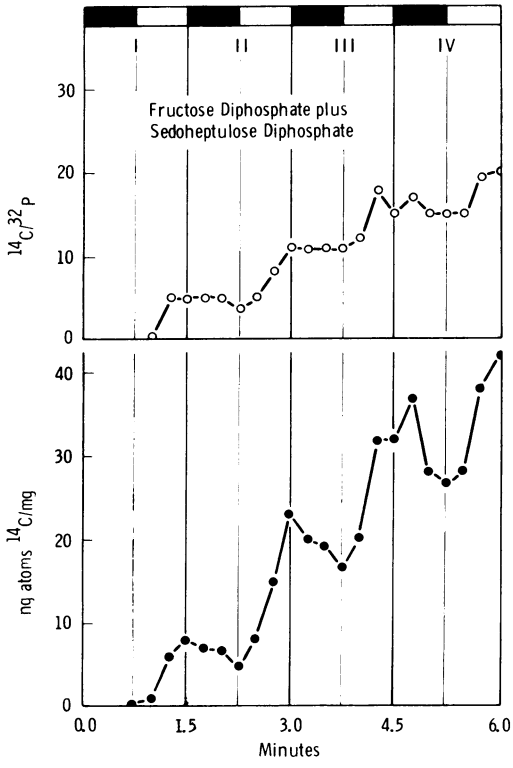

FIG. 3. *$^{14}C/^{32}P$ ratios (top) and concentration of ^{14}C in FDP + SDP (bottom). Data from experiment of Fig. 1.*

in this metabolite in the first 15 s of the dark periods (cycles II to IV). The total $^{14}CO_2$ fixed into PGA in these initial seconds of darkness was nearly equal to the total $^{14}CO_2$ fixed in the dark: i.e., approximately 40 of 48 ng-atoms of ^{14}C per mg of algae. Thus, fixation into PGA is the route of uptake during the first 15 s of darkness.

This conclusion is also supported by the fact that the specific activity of ^{14}C in PGA increased markedly in the initial seconds of the dark periods. As is especially apparent in cycles II to IV, ratios of $^{14}C/^{32}P$ increased at about the same rate in the first 15 s of darkness as in the light. Since the specific activities of the other intermediates were uniformly lower than PGA, rearrangement of previously (light) fixed carbon could not have accounted for the increased specific activities in PGA, in the dark. Only a more highly labeled source, i.e., $^{14}CO_2$ from the gas phase, would have increased the $^{14}C/^{32}P$ ratio in PGA. The slight decrease in $^{14}C/^{32}P$ in PGA during the latter part of the dark periods probably indicates a flow of unlabeled carbon into PGA, coming from the oxidative pentose cycle.

This experiment also makes possible the estimation of relative rates of carbon flow through certain parts of the reductive pentose cycle, in the light and in the dark. In the light periods, newly fixed carbon was observed almost immediately in FDP + SDP and corresponded to a decrease in ^{14}C content in PGA (cycles III to IV;

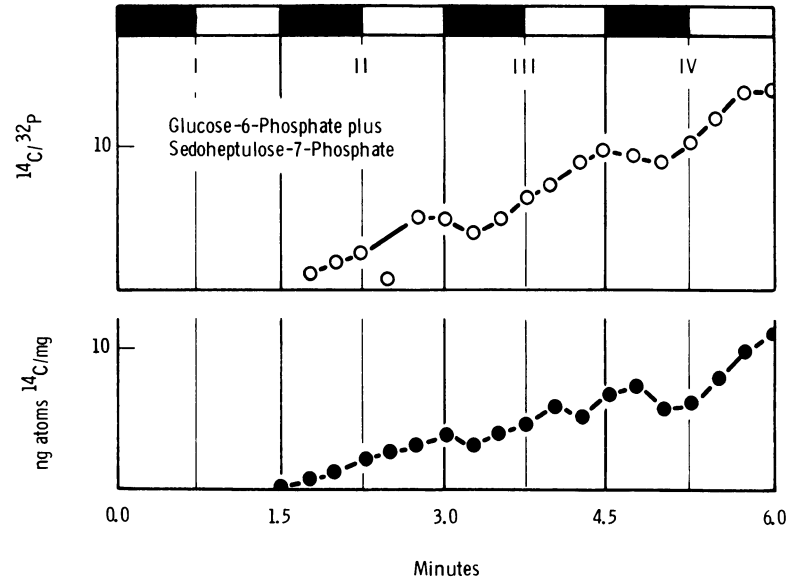

FIG. 4. *$^{14}C/^{32}P$ ratios (top) and concentration of ^{14}C in G6P and S7P (bottom); data from experiment of Fig. 1.*

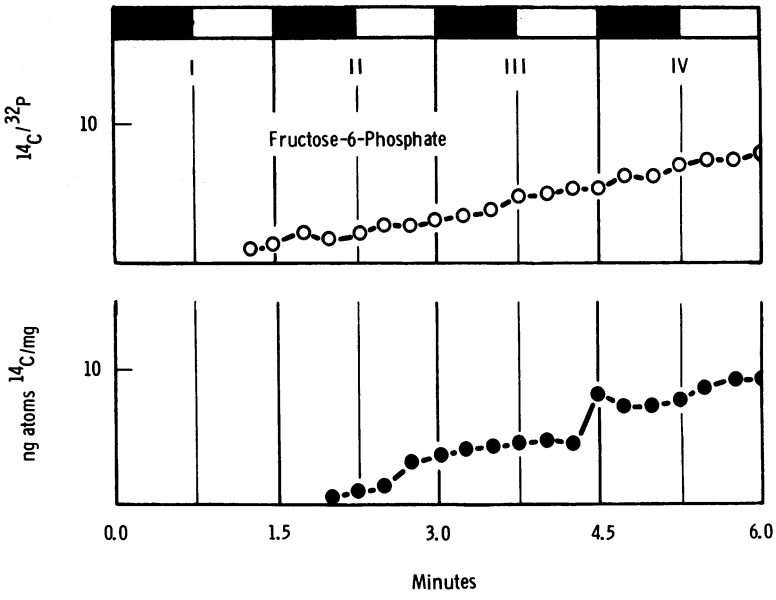

FIG. 5. *$^{14}C/^{32}P$ ratios (top) and concentration of ^{14}C in F6P (bottom); from experiment of Fig. 1.*

Fig. 2 and 3). It should be noted that rates of CO_2 fixation quickly reached steady-state values in these light periods (shown in Fig. 1), showing that carbon flow into PGA was occurring at the maximal rate for this experiment. Thus, fixed carbon in PGA was very quickly converted to FDP + SDP in the light after darkness at a rate in excess of its maximum rates of formation by RuDP carboxylation (i.e., equivalent to the rate of CO_2 fixation). From these observations we conclude that the enzyme sequence PGA-kinase, triose phosphate dehydrogenase, and aldolase synthesized FDP and exchanged label into SDP (via erythrose-4-phosphate) from PGA in the light faster than it was replaced by CO_2 fixation. Since NADPH is required for these conversions (i.e., in the reduction of 1,3-phosphoglycerate to glyceraldehyde-3-phosphate), it can be concluded that the concentration of reduced pyridine nucleotide was sufficient not to limit the flow of carbon in cells transferred from dark to light.

Although the conversion of PGA to FDP and SDP was rapid in the initial seconds of a light period, further conversion of these metabolites to the corresponding sugar monophosphates was slow. The ^{14}C labeling of G6P, S7P, and fructose-6-phosphate (F6P) was very slow relative to that in FDP and SDP (Fig. 4 to 5). This indicates that the reactions mediated by FDPase and SDPase are inactivated in the dark and do not rapidly become activated during the 45-s light period.

Another experiment was designed to test whether conversion of G6P to S7P and F6P depends on light or darkness. In this experiment, [U-^{14}C]glucose was used as the source of label. In both light and dark periods, carbon passed quickly into G6P, S7P, and then from the sugar monophosphates into PGA, FDP, and SDP (Fig. 6 to 8). Thus, during a flashing-light experiment, all of the steps leading from the sugar monophosphates to sugar diphosphates and PGA are fast.

That slow hydrolysis of FDP and SDP was not due to factors other than those produced by flashing the light is shown by the data of Fig. 9. This experiment was carried out with continuous illumination of the cells; otherwise, the conditions were the same as described above. The $^{14}C/^{32}P$ ratio showed that carbon fixed from $^{14}CO_2$ moved quickly throughout the metabolite pools of the reductive pentose cycle, approaching steady-state concentrations by 6 min. More importantly, the specific activities of the monophosphate sugars (made by estimating the number of phosphorous and carbon atoms in a given compound; see Materials and Methods) were approximately equal to those for PGA, FDP, and SDP (after correcting for the number of phosphorous atoms per metabolite). These results should be compared with the comparable data of Fig. 1 to 5 for the flashing-light experiments.

Steady-state experiment: ^{14}C and ^{32}P. In this experiment, both tracers were administered

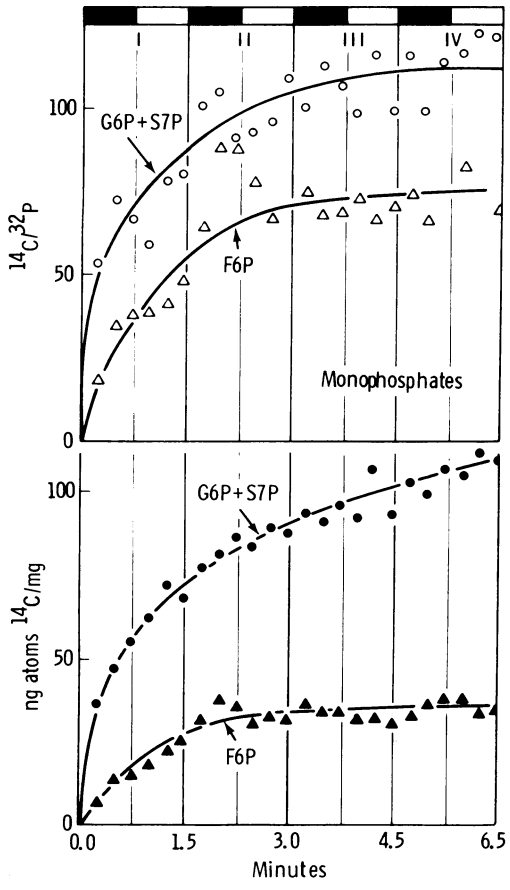

FIG. 6. *$^{14}C/^{32}P$ ratios (top) and ^{14}C concentration (bottom) in G6P and S7P using [^{14}C]glucose as the substrate.*

long enough to achieve steady-state (saturation) labeling of the metabolite pools before the alternating light-dark periods were begun. As can be seen, G6P, F6P, and S7P decreased in concentration fairly rapidly during the dark periods (Fig. 10, top). When cells were reilluminated, approximately 20 s passed before carbon began to flow into these monophosphate pools. It should be noted that in the reductive pentose cycle, the FDP + SDPase reactions must function for carbon to pass from FDP and SDP to the G6P, F6P, and S7P. Thus, the approximately 20-s lag time before the latter compounds were restored to photosynthetic levels was indicative of the time required for reactivation of the FDP + SDPase reactions. This estimate is also in good agreement with initial-velocity data (Fig. 3), which showed accumulation of carbon in FDP and SDP for 20 to 30 s after cells were transferred from dark to light.

The decrease in the concentration of the monophosphate sugars in the dark periods (Fig. 10, top) could be interpreted in a similar way: dark inactivation of FDP + SDPase occurred in approximately 10 s, thus cutting the

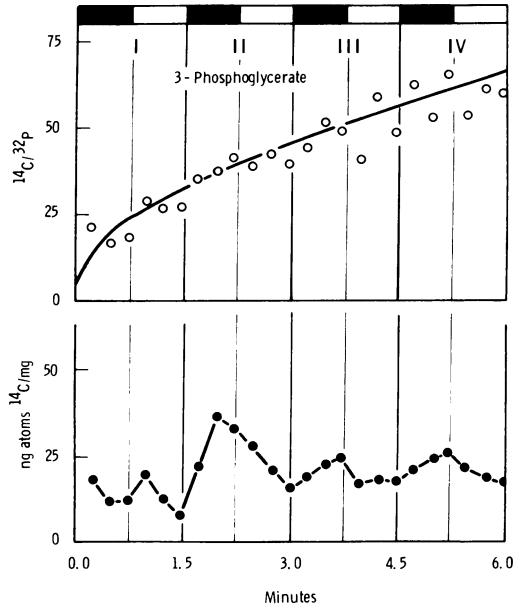

FIG. 7. *$^{14}C/^{32}P$ ratios (top) and ^{14}C concentration (bottom) in PGA. Data from experiment of Fig. 6.*

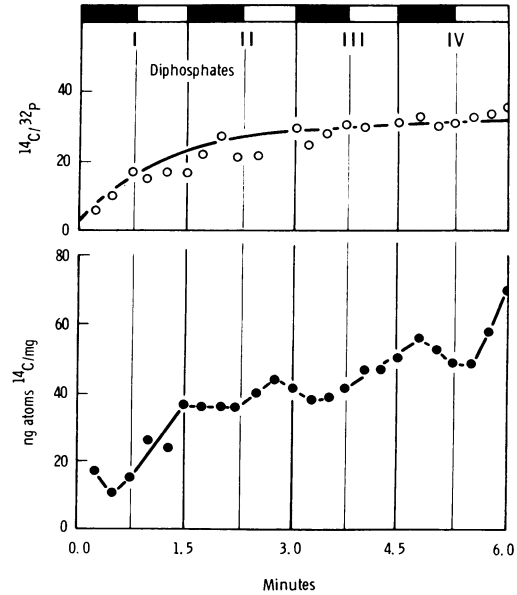

FIG. 8. *$^{14}C/^{32}P$ ratios (top) and ^{14}C concentration (bottom) in FDP and SDP. Data from experiment of Fig. 6.*

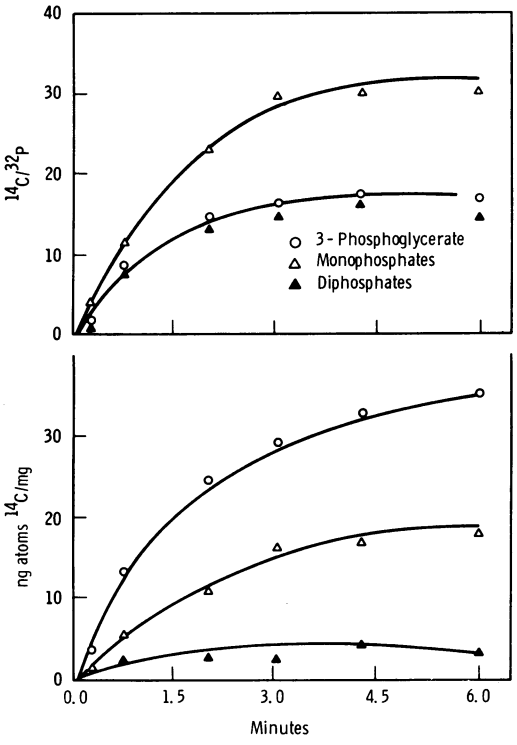

FIG. 9. *$^{14}C/^{32}P$ ratios (top) and ^{14}C concentration (bottom) in a labeling experiment using $^{14}CO_2$ and continuous light.*

major flow of carbon into the monophosphate pools. However, since CO_2 fixation was still active (i.e., 15 to 20 s into the dark: Fig. 1 and 2), the level of carbon in the sugar monophosphates fell as a result of CO_2 fixation and the associated conversion of sugar monophosphates to PGA, via RuDP.

It is significant that, in the dark periods of this flashing-light experiment, the change in RuDP concentration was small in comparison to the rapid expansion of the PGA pool (Fig. 10, bottom). Since RuDP is the major precursor for PGA, it follows that the RuDP pool was turning over rapidly in the initial seconds of darkness. In fact, the RuDP pool must have been replenished several times to account for the total amount of dark-synthesized PGA. Recall that data from one of the initial-velocity labeling experiments (Fig. 1 and 2) showed that the predominant route of PGA synthesis in the early dark periods was via carboxylation of RuDP. Thus, both phosphoribulokinase and the carboxylase were active in the initial seconds of the dark periods.

As seen in the initial-velocity experiments, the overall effect of flashing the light was a steady accumulation of carbon in FDP + SDP (Fig. 11).

As a final point, it should be noted that light-dark and dark-light changes in the adenylate pools required 15 s or less (Fig. 12), which is considerably faster than reactivation of FDP + SDPase or inactivation of CO_2 fixation.

Activity of PRK in cell-free extracts. With 0.5 mM ATP and 0.5 mM Ru5P, PRK was only slightly inhibited by 5 mM adenosine monophosphate (AMP) (Fig. 13). Five millimolar ADP, a 10-fold higher concentration than the substrate ATP, gave approximately 50% inhibition of the enzyme. From the data presented in Fig. 1 and 12, it can be calculated that ATP/ADP ratios in vivo never fell below 0.8 in the flashing-light experiments.

Figure 14 shows the activity of PRK as a function of Ru5P concentration, at fixed ADP or fixed AMP concentrations. Inhibition by 2.0 mM ADP or AMP did not exceed 20 to 30%. These comparatively weak inhibitions by AMP should be contrasted with the strong AMP inhibition of PRK from thiobacilli, hydrogen-oxidizing bacteria (10, 11), and certain photosynthetic bacteria (20). On the other hand, the PRK of *Aphanocapsa* 6714 does not differ greatly from the chloroplast enzyme in its lack of response to inhibition by ADP and AMP (1, 10).

PRK from *Aphanocapsa* 6714 requires a re-

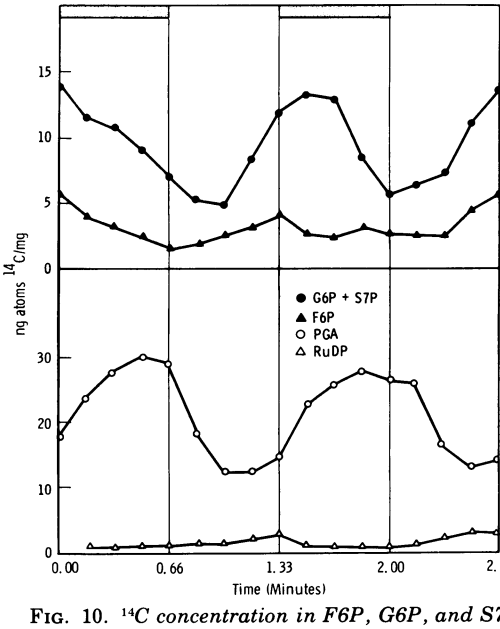

FIG. 10. *^{14}C concentration in F6P, G6P, and S7P (top); PGA and RuDP (bottom) in a steady-state experiment with all pools saturated with respect to tracer.*

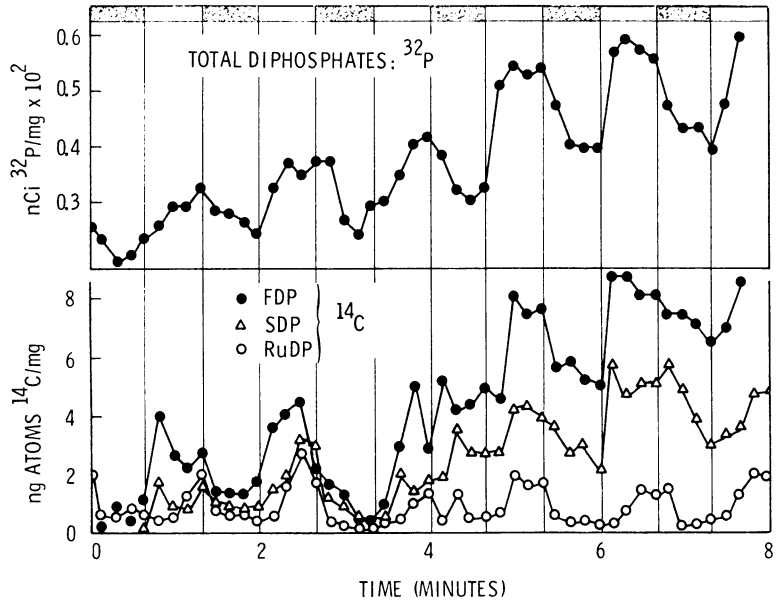

FIG. 11. *^{14}C concentration in FDP, SDP, and RuDP; data from experiment of Fig. 10.*

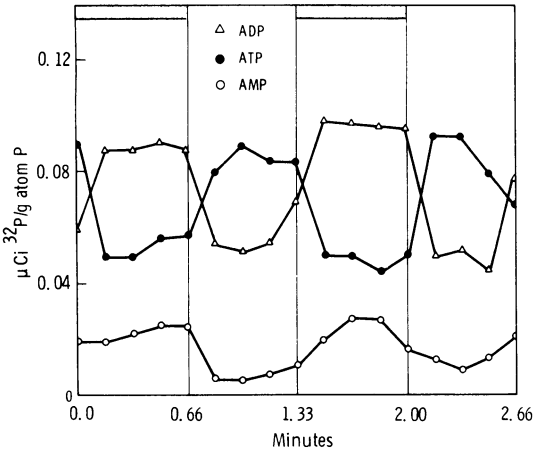

FIG. 12. *^{32}P concentration in ATP, ADP, and AMP; data from experiment of Fig. 10.*

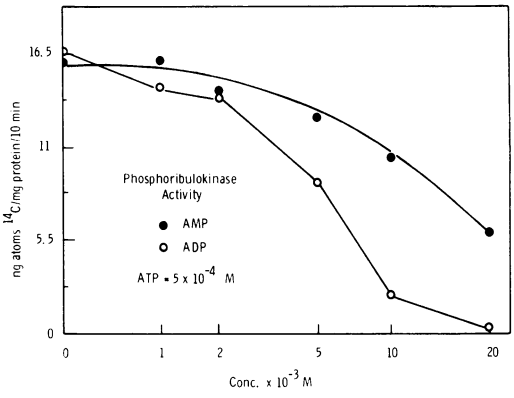

FIG. 13. *PRK activity as a function of AMP or ADP concentration. $NaH^{14}CO_3$ specific activity, 5 μCi/μmol; 3 mg of protein/ml of reaction mix. Ru5P concentration was 5 x 10^{-4} M.*

ducing agent for activity (Table 1). DTT was the most effective reducing agent tested. Glutathione was about one-seventh as effective as DTT; β-mercaptoethanol did not stimulate enzyme activity at the concentrations used. Neither NADPH nor NADH stimulated enzyme activity. This should be contrasted with NADH stimulation of PRK from *Hydrogenomonas facilis* and *Rhodopeudomonas spheroides* (11, 20). On the other hand, stimulation of the chloroplast enzyme by DTT or by light-generated reducing agents has been reported (9), and DTT activation of PRK from the blue-green alga *Anacystis nidulans* has also been demonstrated (5).

DISCUSSION

The reductive pentose cycle in *Aphanocapsa* 6714 is blocked when cells are placed in the dark. This loss of function does not result from a reduced supply of cellular energy; it is more likely the expression of a specific regulatory mechanism that is called into play when cells lose their capacity to carry out noncyclic photo-

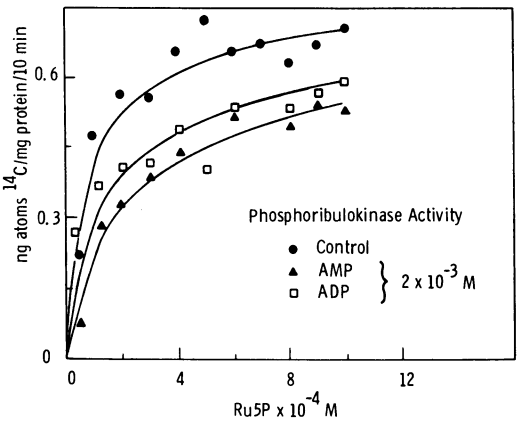

FIG. 14. *PRK activity as a function of Ru5P concentration at fixed AMP or ADP concentrations. Conditions were those of Fig. 13.*

TABLE 1. *Phosphoribulokinase activity versus reducing agents and reduced pyridine metabolites*[a]

Compound	Concn	μg-atoms of ^{14}C/mg of protein per 10 min
DTT	1×10^{-4} M	8.73
GSH	1×10^{-4} M	0.42
Mercaptoethanol	1×10^{-4} M	0.08
NADPH	1×10^{-3} M	0.15
NADH	1×10^{-3} M	0.08
DTT, 10^{-4} M		
No ATP		0.15
DTT, 10^{-4}		
No Ru5P		0.11
No addition		0.24

[a] Conditions were those given in Fig. 13.

electron transport (18).

Previous work showed that one consequence of dark inactivation was the failure of dark cells to fix CO_2, a result probably attributable to inhibition of PRK. This conclusion was based mainly on the observation that dark cells of *Aphanocapsa* 6714 failed to convert measurable quantities of highly labeled [^{14}C]glucose into RuDP (17). It should be noted that failure to synthesize RuDP occurred even though dark cells were actively synthesizing the pentose monophosphate sugars via the oxidative pentose (13, 14, 16). Thus, a supply of ribulose-5-phosphate was present (i.e., the substrate for RuDP synthesis). It is also possible that the carboxylase reaction was inactivated in the dark. However, this result would not have been resolvable in our experiments.

The present work reveals three important features of light-dark flashes on the reductive pentose cycle of *Aphanocapsa* 6714. (i) Dark inactivation of FDP + SDPase was nearly complete, preventing almost all conversion of FDP and SDP to the corresponding monophosphate sugars. (ii) The times required for dark inactivation, and then the subsequent light reactivation of the cycle, were significantly different for CO_2 fixation and hydrolysis of FDP and SDP. (iii) Light-dark effects on the reductive pentose cycle are poorly correlated with changes in the concentrations of the adenylates, arguing against adenylate (or energy charge) control of this pathway.

The first point was established by measuring the initial rates of $^{14}CO_2$ fixation, or [^{14}C]-glucose uptake, into soluble metabolites. From the same experimental data, it was also determined that CO_2 fixation continued for some 15 to 20 s into each of the dark periods of the flashing-light experiments. On the other hand, the FDP + SDPase reactions were rapidly inactivated in the dark. When cells were again placed in the light, the reductive pentose cycle was restored. However, the time required for reactivation of the FDP + SDPase reactions was much longer than for renewal of CO_2 fixation (i.e., a delay of 20 to 30 s versus nearly immediate CO_2 fixation in the light). As a consequence of these different time constants, CO_2 fixed during the flashing-light experiments became trapped in PGA, FDP, and SDP.

Under conditions as they exist in nature, this lack of coordination between these two sites of the reductive pentose cycle should not adversely affect the cell. Inactivation of the cycle would occur once in the evening as cells shift from growth to maintenance metabolism. Reactivation of the cycle would not begin until the following day. Thus, the second point is reasonable in terms of a regulatory mechanism that is used intermittently to block completely or to reactivate photosynthetic metabolism, but not for the fine control of carbon metabolism in the steady state.

The third point, which suggests that the level of adenylates has little or no regulatory effect on the activity of the reductive pentose cycle during light-dark transitions, is based on a general lack of correspondence between inactivation or reactivation reactions and the intracellular concentration level of the adenylates. First, as noted in the initial-velocity experiments, CO_2 fixation took place at an accelerated rate in the initial seconds of the dark periods. At the same time, the concentration of ATP, the ratio of ATP/ADP, and also the energy charge decreased sharply. This is not consistent with adenylate control over CO_2 fixation by the inhibition of PRK, at least as it is

thought to occur in bacteria. In several autotrophic, facultatively autotrophic, and photosynthetic bacteria, AMP (or low energy charge) strongly inhibits the activity of this enzyme (7,10, 11). However, in cell extracts of *Aphanocapsa* 6714, the activity of PRK was insensitive to inhibition by AMP, or product inhibition by ADP (although some inhibition was noted at very high levels of these adenylates, but at ATP/ADP ratios much lower than measured in vivo).

The evidence concerning control of the FDP + SDPase reactions also does not support a role for the adenylates in controlling this part of the reductive pentose cycle. When cells entered a light period (during the flashing-light experiments), CO_2 fixation began almost immediately, and the highest levels of ATP, ATP/ADP, and energy charge were restored within 10 s. Moreover, the levels of NADPH and ATP were sufficiently high to allow rapid conversion of PGA to FDP and SDP. This suggests that neither energy nor reduced pyridine nucleotides were limiting in the environment of the reductive pentose cycle enzymes. Nevertheless, hydrolysis of FDP and SDP was delayed for 20 to 30 s on entering a light period.

In two other photosynthetic systems, *Chromatium* D (13) and spinach chloroplasts (12), the adenylate pools were measured under conditions preventing or limiting growth (in the former case) or CO_2 fixation (in the latter case). For both the bacterium and the chloroplast, energy charge was poorly correlated with metabolism—i.e., high ratios did not stimulate metabolic activity (*Chromatium*) and low ratios did not inhibit activity (chloroplast).

Recently, Ihlenfeldt and Gibson (8) suggested a regulatory role for NADPH, or the ratio of NADPH/NADP, in controlling the rate of CO_2 fixation by *A. nidulans*. They suggested that a principle site of metabolic regulation of CO_2 fixation might be triose phosphate dehydrogenase. This was based partly on the rapid drop in NADPH concentration in cells placed in the dark. In earlier work from this laboratory (16, 17), we obtained kinetic evidence based on incorporation of $^{14}CO_2$ and [^{14}C]glucose, also suggesting a restricted flow of carbon between hexose monophosphate sugars and PGA, which would be consistent with a dark inactivation of triose phosphate dehydrogenase.

However, in more recent work (18) we have shown that NADPH levels in dark cells of glucose-fed *Aphanocapsa* 6714 are rapidly restored to levels equal to or exceeding comparable photosynthetic values. It should be noted that *Anacystis nidulans* is impermeable or only poorly permeable to glucose (19); thus, similar results would not have been obtainable with the latter organism.

As shown in the present study, the rate-limiting steps in the reductive pentose cycle of cells subjected to stress by a flashing-light source are the reactions specific to CO_2 fixation (PRK or both PRK and carboxylase) and the FDP + SDPase reactions. Thus, in *Aphanocapsa* 6714 the light-dark regulation of the reductive pentose cycle is affected mainly at these two reactions via a mechanism that probably does not involve the concentration of NADPH or ratio of NADPH/NADP.

One hypothesis to explain dark inactivation of the reductive pentose cycle is that a reducing agent (or agents) specifically associated with photosynthetic electron transport (but not including NADPH) regulates the activity of this pathway. It has been demonstrated that reduced ferredoxin regulates the activity of FDPase from spinach chloroplasts (4) and, more recently, that a number of reductive pentose cycle enzymes in cell extracts of *A. nidulans* are stimulated by reducing agents (5). Moreover, PRK from spinach chloroplasts is stimulated by DTT or by photosynthetically produced reducing agents (9). These data suggest a similar pattern of metabolic regulation in these oxygen-evolving photosynthetic organisms mediated by photochemically produced reducing agents.

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