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INDUCTION OF RESPIRATORY METABOLISM IN ILLUMINATED *CHLORELLA PYRENOIDOSA* AND ISOLATED SPINACH CHLOROPLASTS BY THE ADDITION OF VITAMIN K_5

G. H. KRAUSE* AND J. A. BASSHAM

Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory, University of California, Berkeley, Calif. (U.S.A.)

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SUMMARY

The effects on photosynthetic metabolism in *Chlorella pyrenoidosa* and in isolated spinach chloroplasts of adding vitamin K_5 have been investigated. The most pronounced effect is the induction of the oxidative pentose phosphate cycle in the light, as indicated by the immediate appearance of 6-phosphogluconic acid upon the addition of vitamin K_5.

The appearance of 6-phosphogluconic acid in isolated spinach chloroplasts demonstrates the operation of the oxidative pentose phosphate cycle in chloroplasts.

The induction of the oxidative cycle is accompanied by inactivation of two enzymes of the photosynthetic carbon reduction cycle, or reductive pentose phosphate cycle. These enzymes are fructosediphosphatase (EC 3.1.3.11) and phosphoribulokinase (EC 2.7.1.19). Some inactivation of ribulosediphosphate carboxylase (EC 4.1.1.39) is also indicated. The inactivation of these enzymes may be related to the normal light–dark regulation of metabolism *in vivo*.

Although it has been reported that addition of vitamin K_5 stimulates cyclic photophosphorylation in broken chloroplasts, such addition in the present experiments caused no increase in the level of ATP in photosynthesizing Chlorella or photosynthesizing intact spinach chloroplasts.

A study of the changes of levels of labeled amino acids and carboxylic acids in photosynthesizing *C. pyrenoidosa* upon the addition of the inhibitor shows an increase in the levels of glutamic and citric acids and a decrease in the levels of aspartic and malic acids. These changes are interpreted as indicating a stimulated increase in the rate of oxidation of pyruvic acid to CO_2 and acetyl-CoA, which may increase the rate of conversion of oxaloacetic acid to citric acid, leading ultimately to the production of glutamic acid.

Abbreviations: DPT, diphosphothiamin; 1,3-P_2-glycerate, 1,3-diphosphoglyceric acid; Fru-6-*P*, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-diphosphate; Glc-6-*P*, glucose 6-phosphate; 3-*P*-glycerate, 3-phosphoglyceric acid; Rib-5-*P*, ribose 5-phosphate; Ribul-5-*P*, ribulose 5-phosphate; Ribul-1,5-P_2, ribulose 1,5-diphosphate; Sed-7-*P*, sedoheptulose 7-phosphate; Sed-1,7-P_2, sedoheptulose 1,7-diphosphate.

* NATO Fellow, 1967–1968. Present address: Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii, U.S.A.

INTRODUCTION

The stimulation of cyclic photophosphorylation by the addition of vitamin K_5 to isolated spinach chloroplasts was reported by ARNON, WHATLEY AND ALLEN[1]. In their studies of the effects of various added inhibitors on the levels of labeled compounds formed by *Chlorella pyrenoidosa* photosynthesizing in the presence of $^{14}CO_2$, GOULD AND BASSHAM[2] found that the addition of vitamin K_5 resulted in a sudden increase in the level of 6-phosphogluconic acid, followed by a later increase of the level of ribose 5-phosphate (Rib-5-*P*). It was concluded that the inhibitor had caused a diversion of electrons during photoelectron transport which resulted in the conversion of NADPH to its oxidized form, as this cofactor was used up for the reduction of diphosphoglyceric acid (1,3-P_2-glycerate) by the carbon reduction cycle. Because of an apparent increase in the level of labeled oligosaccharides and polysaccharides, it was suggested that there was a high level of ATP induced by the addition of vitamin K_5.

In later studies of *C. pyrenoidosa* photosynthesizing under steady-state conditions, both ^{32}P-labeled phosphate and ^{14}C-labeled CO_2 were employed in studies of the transients accompanying the switch from light to dark[3]. It was found that the light–dark transition was accompanied by an appearance of 6-phosphogluconic acid in the dark, indicating the onset of the oxidative pentose phosphate cycle, and by an increase in the ratio of $^{32}P/^{14}C$ labeling in the various metabolic intermediates of photosynthesis and respiration, showing that some pools of such intermediates are common to the processes of photosynthesis and respiration. Moreover, it was found that shortly after the light is turned off, there is a small transient decrease in ATP, after which the level of ATP immediately rises again, presumably due to oxidative phosphorylation.

Other evidence from the light–dark experiments with *C. pyrenoidosa*[4], as well as from studies with isolated chloroplasts[5], has suggested that there is a light–dark regulation of enzymes of the photosynthetic carbon reduction cycle. This permits the photosynthetic cells to carry out efficiently photosynthesis in the light and respiration in the dark, even though intermediate compounds of photosynthesis and respiration are apparently available to the enzymes of both the chloroplasts and the cytoplasm.

In the present experiments, ^{32}P-labeled phosphate has been used together with $^{14}CO_2$ to provide a direct measure of the level of ATP. The effects of vitamin K_5 added at several levels on photosynthetic metabolism in *C. pyrenoidosa*, leading to different degrees of inhibition of photosynthesis, have been studied. Finally, the effects of addition of vitamin K_5 on the metabolism of isolated spinach chloroplasts, photosynthesizing in the presence of $^{14}CO_2$ and ^{32}P-labeled phosphate have been studied. The results are interpreted in terms of the previously reported light–dark regulation.

METHODS AND MATERIALS

C. pyrenoidosa were grown in two ways for these experiments. For the experiments with 1 % or more CO_2, the Chlorella were grown in continuous culture tubes as described by BASSHAM AND CALVIN[6]. For the experiments with air-adapted algae, the Chlorella were grown in batch culture with an added sintered-glass bubbler through which was passed air not enriched in CO_2 (ref. 4). These algae were used after they had been grown for some days with air-level CO_2.

In all experiments with *C. pyrenoidosa*, the algae were harvested from either the

tube culture or the batch culture, centrifuged, and resuspended in a medium consisting only of 0.1 mM KH_2PO_4 and 1 mM KNO_3. The suspension was diluted up to 1 % (v/v) wet-packed algae. 80 ml of this suspension were placed in the steady-state photosynthesis apparatus, as described earlier[7]. Then ^{32}P-labeled phosphate (10–20 mC) was introduced. The algae were permitted to photosynthesize with unlabeled CO_2 for 30–40 min, after which $^{14}CO_2$ was admitted from a closed loop into the closed gas recirculating system. The pH was kept between 5.5 and 6.0 by adding dilute HNO_3 when necessary.

At the beginning of the experiment with algae grown on high levels of CO_2 the level of labeled CO_2 was about 2 %, the specific radioactivity was 33 μC/μmole and the total gas volume was about 1 l. During the course of the experiment, the level of CO_2 did not decline below 1 %.

In the experiments with algae grown in air the volume of the system was 6 l, and the initial level of labeled CO_2 was 0.08 %. This level did not decline below 0.02 % during the course of the experiments. The specific radioactivity of the CO_2 in this case was 50 μC/μmole.

After the addition of $^{14}CO_2$ to the closed gas recirculating system, 1-ml samples of the algae suspension were taken into weighed tubes containing 4 ml of methanol at the times indicated in the figures.

Vitamin K_5 (as the hydrochloride) which had been recrystallized from dilute HCl, dried, and stored in a bottle from which light was excluded, was dissolved in water less than 2 min before its addition to the algae suspension. At the times indicated, enough of this solution was added to make the algae suspension 0.01 or 0.2 mM in vitamin K_5, as indicated in the legends to the figures. Further samples were taken at short intervals just after the addition of the vitamin K_5, and then at longer intervals. During the experiments the rate of photosynthesis before and after addition of vitamin K_5 was determined from the rate of uptake of $^{14}CO_2$ and the known specific activity of the $^{14}CO_2$.

Spinach chloroplasts were obtained by harvesting selected spinach leaves from a nearby farm, transporting the leaves on ice to the laboratory, and isolating the chloroplasts according to methods described previously[8]. Photosynthesis was carried out in a small round-bottomed flask, stoppered with a serum cap. The flask was mounted on a rack which moves in a circular motion in the horizontal plane with the flask immersed in a water bath and illuminated from the bottom with a bank of fluorescent lamps. From previous experiments it was known that the intensity was sufficient for light saturation of CO_2 fixation by the isolated chloroplasts. The suspending medium used contained 0.5 mM phosphate, and to this was added 100 μC of ^{32}P-labeled sodium phosphate which was essentially carrier-free. Enough concentrated suspension of spinach chloroplasts was added to give a suspension with a final volume of 1.0 ml containing about 0.15 mg of chlorophyll. After 4 min preillumination, enough $NaH^{14}CO_3$ solution was added to make the suspension 6 mM in HCO_3^-, with a specific radioactivity of 32 μC/μmole. From time to time, 50-μl samples were withdrawn from the flask and the reaction stopped by addition of 200 μl of methanol. After 7 min photosynthesis with ^{14}C-labeled bicarbonate, vitamin K_5, freshly dissolved in water, was injected into the flask to make a final concentration of 0.04 mM.

For analysis of radioactive compounds, aliquots of the methanolic algae suspension or chloroplast suspension were placed on paper chromatograms which were

developed in two dimensions in phenol–water and in butanol–propionic acid–water, as described previously[6]. After the chromatograms were dried, radioautographs were prepared, and finally, the areas of the paper containing the radioactive spots were cut out and the radioactivity determined. Those radioactive areas of the paper which contained more than one sugar phosphate were eluted with water. The sugar phosphates were hydrolyzed by a phosphatase, and the free sugars obtained were separated by two-dimensional paper chromatography.

For the determination of ^{32}P and ^{14}C radioactivity in individual compounds on the paper, we made use of the semi-automatic spot counter developed by MOSES AND LONBERG-HOLM[9]. This apparatus has been considerably modified to facilitate determination of both ^{32}P and ^{14}C and permit data processing by computer. In this method a special loader is first used to prepare a long strip consisting of two layers of thin mylar, between which are located the pieces of paper cut out from the chromatograms which contained the ^{32}P- and ^{14}C-labeled compounds. As this strip is prepared, pieces of opaque tape are placed in proper position with respect to the filter paper spots so as to be able to control later the operation of a photocell. The strip is wound onto a large reel. This reel and a take-up reel are placed on the automatic spot counter, with the mylar strip running between two opposing large Geiger–Müller counter tubes. In the most recent modification a second pair of opposing Geiger–Müller counter tubes has been added to the apparatus in such a position that it counts one paper spot while the other pair of tubes is counting the next paper spot in the strip. One set of Geiger–Müller tubes has very thin mylar windows which admit most of the β-particles from both ^{14}C and ^{32}P. The second set of Geiger–Müller tubes has aluminum foil windows of such a thickness that they exclude more than 99 % of the ^{14}C β-particles, while admitting about 75 % of the ^{32}P β-particles.

A drive motor turns the take-up reel pulling the strip between the Geiger–Müller tubes. When the two radioactive paper spots are in their proper position between the two sets of Geiger–Müller tubes, the opaque paper at the side of the strip interrupts a light beam to a photocell and the drive motor turning the reel is stopped while the counter scaler is turned on. Counting continues until with each set of tubes a predetermined count or time, whichever comes first, has been reached. The counting is then stopped and the counts from each set of tubes, together with the times, are automatically printed on a data sheet by teletype and at the same time are punched on a paper tape. The drive motor is activated and moves the reel until the next set of spots comes into position. The spot which was previously counted with the thin-window Geiger–Müller tubes is then counted by the aluminum window Geiger–Müller tubes, while a new spot has come between the first pair of tubes.

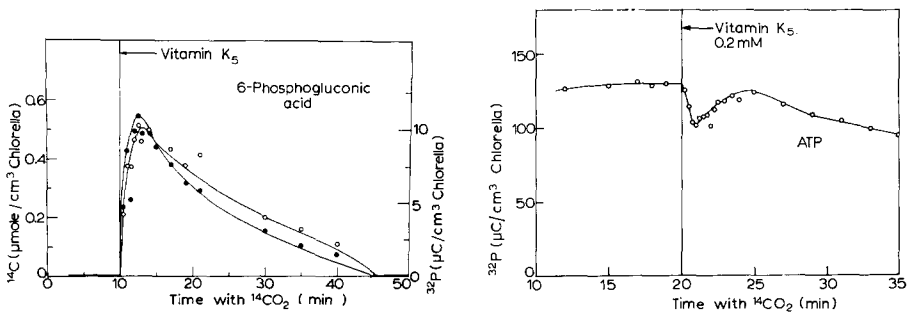
At the end of the counting of all of the spots the counter automatically turns off. Then data related to the sample sizes from which the spots were obtained are typed in by teletype onto the punch tape.

The tape is transferred to a tape-reader which transfers the information into a computer where it is processed according to an appropriate program. The program makes use of previously determined counting constants for carbon and phosphorus in each of the two sets of tubes to calculate the disint./min in each sample; and finally it prints out the μC and μmoles of ^{32}P and ^{14}C, respectively, in each original sample. These are the data which have been plotted *versus* the time of the taking of the original sample and are shown in the figures in RESULTS.

RESULTS

Two experiments were performed with *C. pyrenoidosa*, with vitamin K_5 added to give a final concentration of 0.01 mM. With the *C. pyrenoidosa* grown at 4 % CO_2 (Expt. A) 65 % inhibition of photosynthesis was obtained upon addition of the inhibitor. With the algae grown in air and given 0.08 % $^{14}CO_2$ (Expt. B), an inhibition of 20 % was obtained. In two experiments, C and D, a level of 0.2 mM vitamin K_5, added to Chlorella grown in air and given an initial concentration of 0.08 % $^{14}CO_2$ caused full inhibition. In Expt. E with isolated spinach chloroplasts, 0.04 mM vitamin K_5 was administered and gave an inhibition estimated at 80 to 90%.

In Expts. A and B (0.01 mM vitamin K_5) no change in the rate of labeling of ATP could be detected upon the addition of the inhibitor. The most pronounced effect seen was the sudden appearance of 6-phosphogluconic acid, which is shown in Fig. 1 for Expt. A. The curve of 6-phosphogluconic acid in Expt. B was almost identical with respect to both the height of the peak and the time for its formation and disappearance. Other dramatic changes in intermediates of the photosynthetic carbon cycle were



Figs. 1–15. Transient changes in levels of labeled compounds induced by addition of vitamin K_5 during photosynthesis in the presence of ^{32}P-labeled phosphate and $^{14}CO_2$.

Fig. 1. ^{32}P (○) and ^{14}C (●) labeling of 6-phosphogluconic acid in Expt. A (addition of 0.01 mM vitamin K_5 to *C. pyrenoidosa* grown in 4 % CO_2 in air).

Fig. 2. ^{32}P label of ATP in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

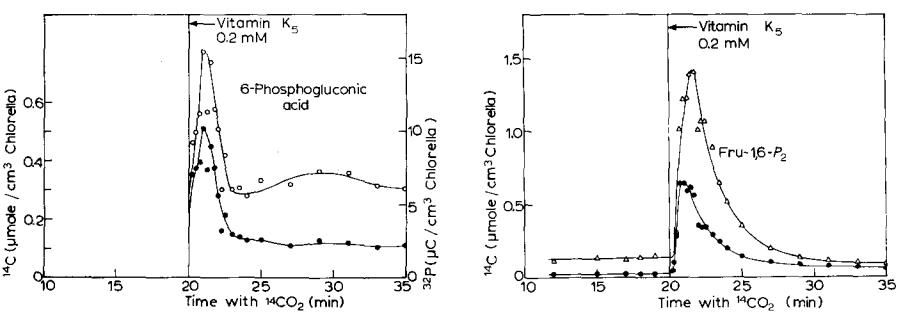


Fig. 3. ^{32}P (○) and ^{14}C (●) label of 6-phosphogluconic acid in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

Fig. 4. ^{14}C labels of Fru-1,6-P_2 (△) and Sed-1,7-P_2 (●) in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

absent in Expt. B. The transient changes in levels of intermediates of the carbon cycle in Expt. A, where there was 65 % inhibition, were generally the same but less dramatic than those seen in Expts. C and D, which will be shown in more detail.

The inhibitor-induced results in Expts. C and D, where there was 100 % inhibition, were essentially identical in every case. Transient changes in the level of ATP are shown in Fig. 2, where it is seen that the ^{32}P label of ATP declines momentarily and then comes back to approximately its steady-state level. The percentage change in ATP level seems, in any event, to be too small to account for other effects to be described. The change in the level of ^{14}C- and ^{32}P-labeled ADP was approximately the inverse of that shown for ATP, except that the percentage change was somewhat greater.

The level of 6-phosphogluconic acid is shown in Fig. 3, where it is seen that following the initial rise and fall the level does not decline to zero.

Fig. 4 shows the sudden increase and decline in the levels of sedoheptulose 1,7-diphosphate (Sed-1,7-P_2) and fructose 1,6-diphosphate (Fru-1,6-P_2). Fig. 5 gives the decrease in levels of fructose 6-phosphate (Fru-6-P). Glucose 6-phosphate (Glc-6-P) and sedoheptulose 7-phosphate (Sed-7-P) (not shown) also decline.

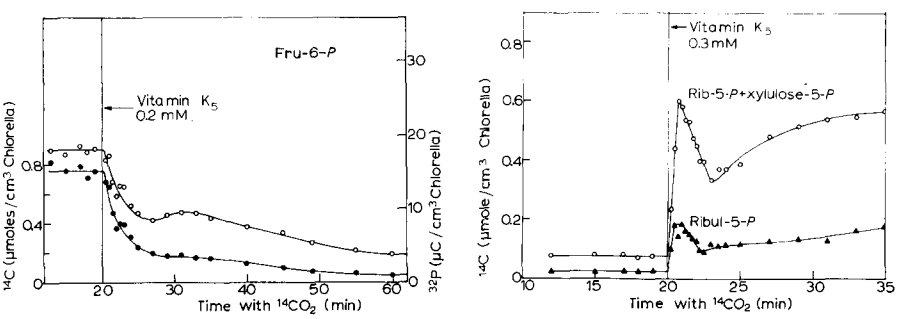


Fig. 5. ^{32}P label (○) and ^{14}C label (●) of Fru-6-P in Expt. C (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

Fig. 6. ^{14}C label of Rib-5-P *plus* xylulose 5-phosphate (○) and ^{14}C label of Ribul-5-P (▲) in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

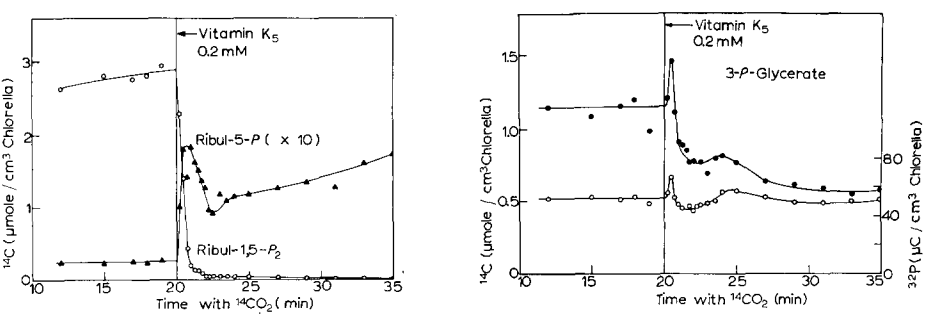


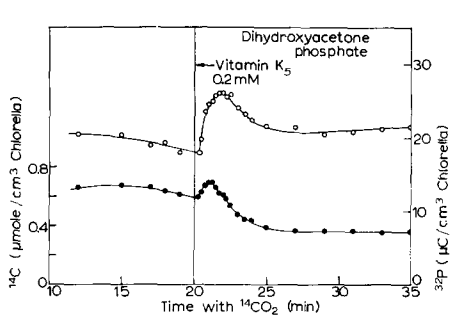
Fig. 7. ^{14}C label of Ribul-1,5-P_2 (○) and of Ribul-5-P (× 10) (▲) in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

Fig. 8. ^{32}P label (○) and ^{14}C label (●) of 3-P-glycerate in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

Changes in the levels of pentose phosphates in Expt. D are shown in Fig. 6. Upon addition of the inhibitor, there was a very rapid rise, a subsequent fall, and then a final rise to a high level.

Fig. 7 shows the drop in the level of ribulose 1,5-diphosphate (Ribul-1,5-P_2) in the same experiment. Note, however, that the level of Ribul-1,5-P_2 does not decline to zero, especially in the first 5 min after the addition of inhibitor. For comparison, the level of ribulose 5-phosphate (Ribul-5-P) is shown. Fig. 8 shows that the level of labeled phosphoglyceric acid (3-P-glycerate) rises momentarily and then drops to a new steady-state level. Fig. 9 indicates the level of the only other measurable intermediate of the carbon reduction cycle, dihydroxyacetone phosphate, which rises momentarily but to a lesser extent than Fru-1,6-P_2 and Sed-1,7-P_2.

Fig. 10 shows that the level of citric acid rises rapidly and then falls upon the addition of inhibitor, while Fig. 11 shows a large decline in the level of labeled malic acid. Fig. 12, from the same experiment, shows that addition of inhibitor causes an increase in the level of glutamic acid and a decrease in the level of aspartic acid. Fig. 13 shows the levels of glycine and alanine nearly unchanged.



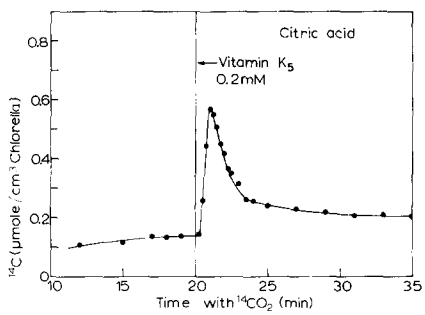
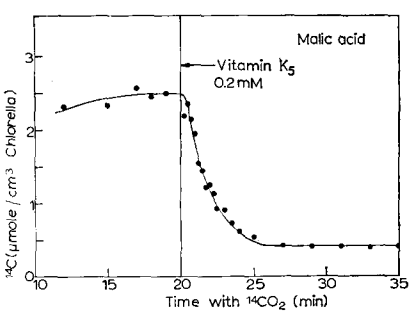


Fig. 9. ^{32}P label (○) and ^{14}C label (●) of dihydroxyacetone phosphate in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

Fig. 10. ^{14}C label of citric acid in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).



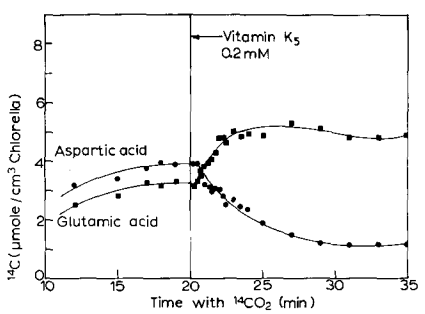


Fig. 11. ^{14}C label of malic acid in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

Fig. 12. ^{14}C label of aspartic acid (●) and glutamic acid (■) in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

The labeling of 6-phosphogluconic acid in isolated spinach chloroplasts (Expt. E) is shown in Fig. 14 and is seen to be quite similar to the appearance of 6-phosphogluconic acid in the algae. The levels of Glc-6-*P* and Sed-7-*P* decline correspondingly. In Fig. 15 it is seen that the level of ATP remains virtually unchanged in the isolated chloroplasts upon the addition of vitamin K_5. In other results, not shown, the levels of Fru-1,6-P_2 and Sed-1,7-P_2 became constant after addition of vitamin K_5, in contrast to the case with the algae. Another difference was seen in the level of 3-*P*-glycerate, which rose and did not subsequently decline in isolated chloroplasts.

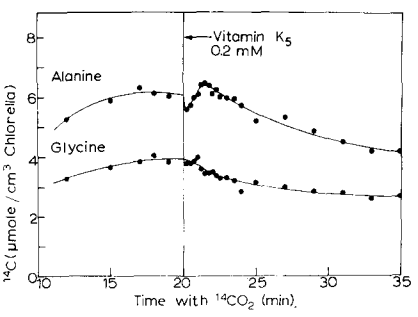


Fig. 13. ^{14}C label of alanine (upper curve) and of glycine (lower curve) in Expt. D (addition of 0.2 mM vitamin K_5 to *C. pyrenoidosa* grown in air).

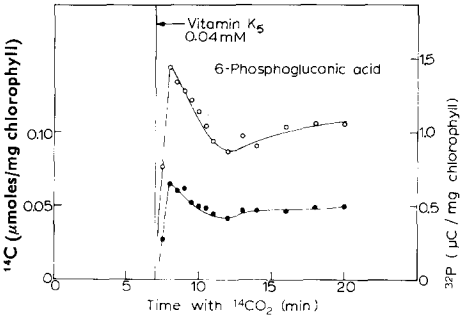


Fig. 14. ^{32}P (○) and ^{14}C (●) label of 6-phosphogluconic acid in Expt. E (addition of 0.04 mM vitamin K_5 to isolated spinach chloroplasts).

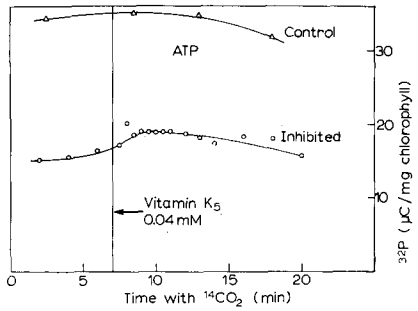


Fig. 15. ^{32}P label of ATP in Expt. E (addition of 0.04 mM vitamin K_5 to isolated spinach chloroplasts). Upper curve (△) shows the level of ATP in a separate control experiment, while lower curve (○) shows the level of ATP in a different flask before and after addition of vitamin K_5. The twofold variation in the level of labeled ATP in the two different flasks of the same original preparation of spinach chloroplasts represents a variation in the steady-state level from one flask to another, which is commonly encountered.

DISCUSSION

The most immediate effect and the one seen at the lowest concentrations of added inhibitor and the lowest degrees of inhibition is the formation of 6-phosphogluconic acid upon addition of the inhibitor. This formation of a key intermediate in the oxidative pentose phosphate cycle suggests a primary effect of the addition of vitamin K_5 is a diversion of electrons from the photoelectron transport system, thus

leading to a cessation of the reduction of $NADP^+$ to NADPH by reduced ferredoxin. In terms of the commonly accepted two-light reaction scheme[10], this would suggest that electrons from the reducing side of the light reaction I are short-circuited by vitamin K_5 back to the intermediate electron transport chain between light reaction I and light reaction II.

However, in the case of a system which is photosynthesizing with CO_2, either whole cells or isolated chloroplasts, results of this present study show that this does not lead to an increase in the ratio of endogenous ATP/ADP. Even in the presence of high levels of vitamin K_5 (Expts. C and D), no increase, but rather a transient decrease in ATP level is seen, despite the fact that less utilization of ATP is expected after cessation of CO_2 uptake. Possibly this suggests that cyclic photophosphorylation which has been previously observed with isolated chloroplasts, added vitamin K_5, and added ADP[1], may only proceed to an extent necessary to maintain a certain ratio of ATP/ADP.

The sudden appearance of appreciable amounts of labeled 6-phosphogluconic acid in isolated spinach chloroplasts upon the addition of vitamin K_5 (Fig. 14) clearly proves the operation of the oxidative pentose phosphate cycle in the chloroplasts. That this oxidative cycle operates in chloroplasts *in vivo* had been proposed[3] on the basis of kinetic light–dark studies and vitamin K_5-induced transient changes[2] in *C. pyrenoidosa*. Recently, HEBER, HALLIER AND HUDSON[11] found that all enzymes required for operation of this cycle are present in isolated chloroplasts.

As in these previous studies, the present results show in those cases where both ^{32}P and ^{14}C label was determined, that after addition of the vitamin K_5 the ratio of ^{32}P label/^{14}C label increases with time. This is taken as an indication of the oxidative conversion of stored sugars which are not fully labeled with ^{14}C to intermediates of the photosynthetic carbon reduction cycle. The change in $^{32}P/^{14}C$ label is either absent or only barely visible in the chloroplast experiment. This is due, no doubt, to the lack of appreciable quantities of stored sugars, and the consequent formation, upon addition of vitamin K_5, of 6-phosphogluconic acid and other metabolites from intermediates of the photosynthetic carbon cycle which are already equally labeled with respect to ^{14}C and ^{32}P.

Functioning of the oxidative pentose phosphate cycle upon addition of vitamin K_5 would be expected to result in an increased production of pentose monophosphate, due to the increased rate of oxidation of 6-phosphogluconic acid to CO_2 and Ribul-5-*P*. However, the rapid increase in the levels of all pentose monophosphates, together with the rapid decline in the level of Ribul-1,5-P_2, and the fact that the level of ATP does not decline more than 20 %, is a convincing indication that there is an inhibition of the reaction which converts Ribul-5-*P* and ATP to Ribul-1,5-P_2.

Previous light–dark studies[3,4] and studies with fatty acid inhibitors[12] had provided strong evidence of a light–dark regulation of the reaction converting Ribul-1,5-P_2 and CO_2 to 3-*P*-glycerate and the reaction which converts Fru-1,6-P_2 and Sed-1,7-P_2 to their respective monophosphates. These two reactions are mediated by ribulose diphosphate carboxylase (EC 4.1.1.39) and by hexosediphosphatase (EC 3.1.3.11), respectively.

The inactivation of phosphoribulokinase (EC 2.7.1.19) was seen but not recognized by GOULD AND BASSHAM[2] in their previous study on the effects of vitamin K_5 added to *C. pyrenoidosa*. The rise in the level of Rib-5-*P* and the fall in the level of

Ribul-1,5-P_2 at a time when the level of ATP was presumed to be increasing was taken as an indication that the formation of Rib-5-P might be at a site 'separated from the carbon reduction cycle'. The observation that 6-phosphogluconic acid is formed even in isolated chloroplasts in the present study (Fig. 14) clearly rules out that interpretation of the earlier results. A study with fatty acid inhibitors[12] showed also a rise in the level of Rib-5-P and Ribul-5-P and a decline in the level of Ribul-1,5-P_2 upon addition of the inhibitor. In that case, however, the level of ATP was greatly diminished upon addition of inhibitor, so that the inactivation in the conversion of Ribul-5-P and ATP to Ribul-1,5-P_2 could be attributed to the lower level of ATP.

The previous light–dark study[4] which gave evidence for the dark inhibition of the carboxylase and diphosphatase reactions did not clearly reveal a dark inactivation of the phosphoribulokinase. The level of Ribul-1,5-P_2 did decline rapidly, but the changes in levels of pentose monophosphates were barely beyond the level of experimental error. This problem is being reinvestigated using more sensitive techniques. However, we must keep in mind the fact that the level of pentose monophosphates is determined not only by its rate of conversion to Ribul-1,5-P_2 but also during photosynthesis by the rate of its formation from hexose and triose monophosphates and during oxidative metabolism by the rate of formation from oxidation of 6-phosphogluconic acid and conversion to hexose and triose monophosphates. It is conceivable that during the transition period from light to dark these several rates could change in such a way to produce only minor transient changes, such as have been observed[4].

Figs. 4 and 5, in which addition of vitamin K_5 causes a dramatic transient increase in the levels of Fru-1,6-P_2 and Sed-1,7-P_2 and a decrease in the level of Fru-6-P, clearly indicate that vitamin K_5 has induced an inactivation of the hexosediphosphatase, which was previously observed during the transition from light to dark[3,4] and upon addition of fatty acids[12]. As in those previous studies, the formation of Fru-1,6-P_2 is presumed to be due to a reaction of ATP and Fru-6-P in the presence of phosphofructokinase (EC 2.7.1.11), which must be activated at the same time as the diphosphatase becomes inactive. Probably this formation of Fru-1,6-P_2 occurs in the cytoplasm since HEBER, HALLIER AND HUDSON[11] have found that phosphofructokinase is not present in the chloroplasts. This would explain our finding that in isolated chloroplasts the level of Fru-1,6-P_2 stops increasing and remains constant after addition of vitamin K_5.

Thus, addition of vitamin K_5, which stimulates the operation of the oxidative pentose phosphate cycle as indicated by appearance of 6-phosphogluconic acid, also brings on the inhibitory regulation of two enzymes, phosphoribulokinase and diphosphatase. This inhibition permits respiratory metabolism to operate efficiently.

Inhibition of phosphoribulokinase prevents the conversion of Ribul-5-P and ATP to Ribul-1,5-P_2. Inhibition of hexosediphosphatase prevents the hydrolysis of Fru-1,6-P_2. Since it appears that Fru-1,6-P_2 can diffuse readily between chloroplasts and cytoplasm[13], diphosphatase in the chloroplasts and phosphofructokinase in the cytoplasm would together act as an ATPase.

In chloroplasts in the dark the hexose monophosphates are recycled through the oxidative pentose phosphate cycle, producing CO_2, NADPH, and glyceraldehyde phosphate. Presumably the latter is converted with triosephosphate dehydrogenase (EC 1.2.1.13) and phosphoglycerate kinase (EC 2.7.2.3) to 3-P-glycerate, ATP, and

NADPH. In this way the chloroplasts *in vivo* can produce NADPH inside the chloroplast for biosynthetic reactions in the dark.

Evidence was found by HEBER AND SANTARIUS[14] that NADPH and $NADP^+$ are not transported from the chloroplasts, whereas ATP can diffuse in and out of the chloroplast. Thus, *in vivo* ATP produced by oxidative phosphorylation in the dark can diffuse into the chloroplasts and, together with NADPH produced by the oxidative pentose phosphate cycle in the chloroplasts, supply the necessary energy and reducing power for biosynthetic reactions, such as the conversion of stored sugars to fats[3].

The photosynthetic carbon reduction cycle, with proposed sites of inhibition by vitamin K_5, by fatty acids, and by darkness, is shown in Fig. 16. A portion of the oxidative pentose phosphate cycle, including Glc-6-*P* and 6-phosphogluconic acid, is also shown.

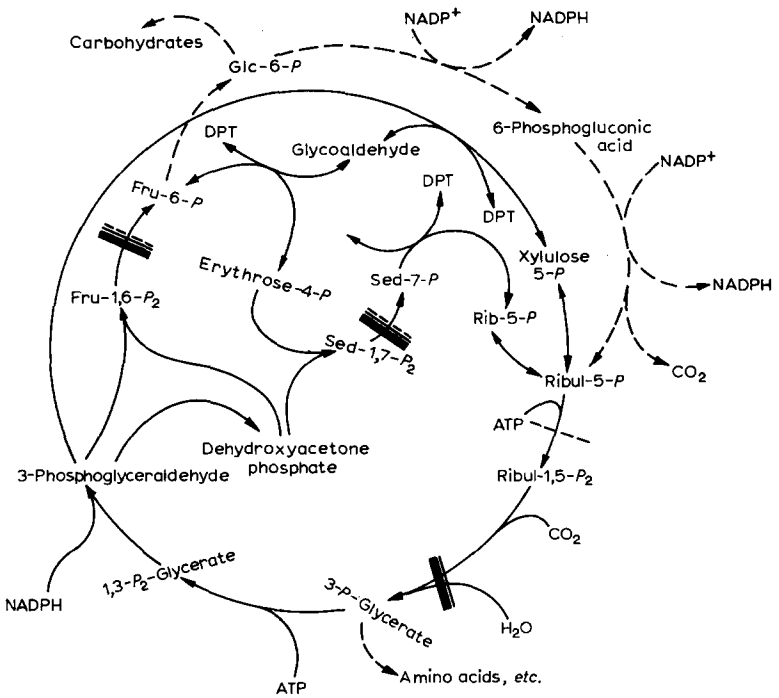


Fig. 16. The photosynthetic carbon reduction cycle and proposed sites of inhibition. Thick bar indicates proposed sites of light–dark inhibition; short, thin solid line indicates fatty acid inhibition sites; and short dotted line indicates postulated sites of inhibition with vitamin K_5. Also shown (dotted pathway) is portion of oxidative pentose phosphate cycle through Glc-6-*P* and 6-phosphogluconic acid.

Light–dark transient studies and studies with fatty acids gave evidence of inhibition of ribulosediphosphate carboxylase. In the present study, with added vitamin K_5, the level of Ribul-1,5-P_2 in several experiments does not decline below measurable limits, but the evidence for inactivation of the enzyme is not conclusive.

Even though NADPH cannot apparently diffuse from the chloroplasts *in vivo*, it is clear from the results in Figs. 10–13 that there is an indirect effect on metabolism in

the cytoplasm caused by cessation of the production of reducing power from the photoelectron transport system. The rise in citric acid upon the addition of vitamin K_5 (Fig. 10) while malic acid (Fig. 11) decreases, and the rise in glutamic acid while aspartic acid decreases (Fig. 12) indicate such an effect. These results suggest that upon the addition of vitamin K_5 there is an accelerated condensation of acetyl-CoA with oxaloacetic acid, giving citric acid and, ultimately, glutamic acid. The steady-state level of the oxaloacetic acid, formed by carboxylation of phosphoenolpyruvic acid, decreases, resulting in the decrease in the formation of malic acid and aspartic acid. Moreover, the curve for glycine, shown in Fig. 13, indicates that there is no general stimulation, or inhibition, of the production of amino acids upon the addition of vitamin K_5.

The increase in glutamic acid is similar to that reported by CALVIN AND MASSINI[15] in earlier studies of light–dark transient changes. ATKINSON[16] has suggested that the explanation for the increased flow of carbon into the citric acid cycle might lie in the strong effect of ATP on the affinity of citrate synthase (EC 4.1.3.7) for acetyl-CoA, observed with this enzyme from yeast[17]. However, we now know that in the light–dark transition[3,4], as well as in the vitamin K_5-induced changes described in the present report, the level of ATP does not change greatly. In both cases there is a small transient drop in ATP concentration before it comes back to the same level. In the dark, the level is restored by oxidative phosphorylation; possibly this is also the case when vitamin K_5 is added.

It seems likely that the metabolic regulation of key enzymes in the green plant cells is produced by a somewhat different mechanism. The regulation of fructose-diphosphatase by AMP in non-photosynthetic tissue is well documented. In green cells, although fructosediphosphatase is subject to light–dark regulation, the isolated enzyme is unaffected by changes in level of AMP[18]. Its activity is affected by changes in level of Mg^{2+}, and of $MgP_2O_7^{2-}$, $MgADP^-$, and $MgATP^{2-}$ (in increasing order of inhibition).

The regulation of ribulosediphosphate carboxylase may be to some extent determined by pH and the level of Mg^{2+} in the chloroplasts[19].

The regulation of phosphoribulokinase, suggested by results in the present report, may be due to changes in redox potential within the chloroplasts. Addition of vitamin K_5 apparently stops the flow of electrons to $NADP^+$, so that NADPH becomes oxidized. GIBBS, ELLYARD AND LATZKO[20] found that phosphoribulokinase activity is easily lost in O_2 and can be preserved with sulfhydryl reducing agents.

It is not possible to say at present whether the level of Mg^{2+}, redox potential, or other factors are likely to be responsible for the changes observed in the levels of di- and tricarboxylic acids.

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