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SUMMARY

Using isolated spinach chloroplasts capable of high rates of photosynthesis with $^{14}CO_2$, we have studied the rates of $^{14}CO_2$ fixation during the sequence light-dark-light, with and without added cofactors, ribose-5-phosphate, and ribulose-1,5-diphosphate. Except for a small fixation of $^{14}CO_2$ during the first 3 min after the light is turned off, no significant fixation of $^{14}CO_2$ is observed in the dark with these chloroplasts, even when ATP and ribose-5-phosphate are added. Moreover, the addition of ATP does stimulate the continued formation of the carboxylation substrate, ribulose-1,5-diphosphate, in the chloroplasts, because subsequent radiochromatographic analysis of aliquot samples of the chloroplast suspensions show the level of ribulose-1,5-diphosphate to be about as high in the dark with added ATP as it was in the first light period, when the fixation rate was high. When the light is again turned on, the fixation rates are diminished as compared with those of the first light period in all cases. The levels of ribulose-1,5-diphosphate are much higher during the second light period than in the first, and are enhanced by the addition of ATP. These results provide additional evidence for the previously proposed light activation of the carboxylation reaction of photosynthesis. Furthermore, it appears that the primary cause of decreased rate of fixation with time in these highly active isolated chloroplasts is loss of activity of the carboxylation enzyme. The principal cause of this loss may be loss of the light activation.

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Kinetic studies of the levels of ^{14}C- and ^{32}P-labeled intermediate compounds of the carbon reduction cycle of photosynthesis[1] revealed two major points of metabolic regulation.[2] One of these points is the diphosphatase reaction which converts fructose-1,6-diphosphate (Fru-1,6-P_2) and sedoheptulose-1,7-diphosphate (Sed-1,7-P_2) to their respective monophosphates and inorganic phosphate (P_i). The other control point is the carboxylation reaction which converts ribulose-1,5-diphosphate (Ribul-1,5-P_2) and carbon dioxide to two molecules of 3-phosphoglyceric acid.

The enzyme which catalyzes the carboxylation reaction is ribulose diphosphate carboxylase, (3-phospho-D-glycerate carboxy-lyase, dimerizing, 4.1.1.39). The only known activators for the isolated enzyme are Mg^{++} ion (which can be replaced by Ni^{++} ion) and sulfhydryl compounds such as glutathione or cysteine.[3] However, the effects of sulfhydryl compounds vary with the age and activity of the enzyme, and the effects can be replaced by ethylenediamine tetraacetic acid.[3]

Recent studies[4] with isolated spinach chloroplasts gave results which indicated that the carboxylation reaction virtually stops in the dark, even though there remain adequate amounts of Ribul-1,5-P_2 and CO_2 for the reaction to continue. When the light was turned off, uptake of $^{14}CO_2$ soon ceased and the level of Ribul-1,5-P_2 dropped to about one-half its level in the light and then remained constant. If the enzyme were still active, the level of Ribul-1,5-P_2 should have continued to drop. When the light was turned on, the level of Ribul-1,5-P_2 rose rapidly to a very high value, then fell to the steady-state level. This "overshoot" suggests that the carboxylation reaction was not fully activated for some seconds after the light was turned on, whereas the formation of Ribul-1,5-P_2 from ribulose-5-phosphate (Rib-5-P) and ATP produced from the light reactions commenced almost immediately.

In order to test further these interpretations, we have investigated the effect of added ATP, NADPH, and Ribul-1,5-P_2 on the incorporation of $^{14}CO_2$ by

chloroplasts in the dark and in the light. From studies of the distribution of photosynthetic intermediate compounds between isolated chloroplasts and the solution in which they are suspended, we know that ATP diffuses rapidly in and out of the isolated spinach chloroplasts[5] when they are prepared and suspended according to conditions used in these and previous experiments.[6] There is also evidence that Rib-5-P diffuses rapidly out of the chloroplasts in our experiments. Using a less active but somewhat similar chloroplast preparation, Walker[7] found that Rib-5-P stimulated $^{14}CO_2$ uptake, thus indicating that in his system added Rib-5-P does come in contact with the enzymes of the carbon reduction cycle.

Even with very active preparations of isolated spinach chloroplasts (fixation rates approaching in vivo rates based on chlorophyll content), addition of Rib-5-P without preillumination causes a small stimulation of $^{14}CO_2$ uptake during the first 3 min when the rate would not otherwise have reached its maximum. This indicates that Rib-5-P can enter even our most active isolated chloroplasts. However, a preillumination period of 3 min prior to addition of $^{14}CO_2$ eliminates the induction period, and addition of Rib-5-P in this case causes no stimulation.

If ATP and Rib-5-P can enter these chloroplasts, and if the enzymes catalyzing the conversion of these metabolites to Ribul-1,5-P_2 are active, isolated chloroplasts should form Ribul-1,5-P_2 in the dark from ATP and Rib-5-P. Thus, it should be possible to investigate the carboxylation reaction in the dark in isolated chloroplasts. Also, it was of interest to see if added Ribul-1,5-P_2 could stimulate $^{14}CO_2$ fixation in the dark.

There is no evidence that $NADPH_2$ can enter intact spinach chloroplasts, but the effect of adding $NADPH_2$ was tested beacuse it has been reported that broken chloroplasts, or chloroplast extracts, can fix $^{14}CO_2$ if supplied in the dark with ATP and $NADPH_2$.[8] Since the chloroplast preparations used in the

present study included 10-30% broken chloroplasts, one might possibly have expected some fixation of $^{14}CO_2$ in the presence of added ATP and $NADPH_2$ by reactions outside the intact chloroplasts.

One of the differences between the carboxylation activity of spinach chloroplasts isolated according to our method, and the isolated carboxylation enzyme is a much lower apparent Michaelis constant for the chloroplasts[6] than for isolated enzyme.[3] Therefore the effect of higher concentrations of $H^{14}CO_3^-$ on dark fixation was studied.

EXPERIMENTAL

Chloroplasts were prepared from fresh spinach as described previously.[6] In the first experiment, an amount of chloroplast suspension containing 0.063 mg chlorophyll was added to each of three small flasks in the rack over the illumination table.[6] After 3 min of illumination of the stoppered flasks, 3.75 μmoles of $NaH^{14}CO_3$ (35.2 μC/μmole) was added to each flask. After 6 min photosynthesis with $H^{14}CO_3^-$, the flasks were darkened, and immediately thereafter the following additions were made: (a) 1 μmole NaCl, (b) 0.5 μmole ATP and 0.5 μmole NaCl, (c) 0.5 μmole ATP and 0.5 μmole Rib-5-P. In each case, the final concentration of each added cofactor or metabolite was 1.0 mM. After 5 min of darkness, the chloroplasts were again illuminated. During the first period of photosynthesis, during the dark period, and during the second period of photosynthesis, 50 μl samples were taken from the original 500 μl volume and biochemical activity stopped with addition of 200 μl of methanol to each sample. Total ^{14}C fixed into stable compounds was determined,[6] and the samples were analyzed by two-dimensional paper chromatography and radioautography. The ^{14}C content of photosynthetic intermediate compounds was determined,[6] and fixation rate and amounts of labeled intermediate compounds were calculated from the known specific radioactivity of the $H^{14}CO_3^-$ used.

In Experiments 2, 3, and 4, similar conditions were used, except as indicated in Table I. The additions made at the beginning of the dark period (see Table I) were such as to give final concentrations of 1.0 mM for each added cofactor or metabolite. Each of the four experiments was carried out with a different chloroplast preparation. Hence the chlorophyll contents (last column, Table I) and the control rates were different in each experiment. In each control, and in the other flasks as needed, 1 or 2 mM NaCl was added to keep the total ionic strength about constant within each experiment.

In Experiments 3 and 4, the concentration of $H^{14}CO_3^-$ was raised to 16 mM and to 50 mM, respectively.

In Experiment 5, identical chloroplast suspensions in three flasks were first allowed to photosynthesize for 6 min with unlabeled HCO_3^-. The light was then turned off, and $H^{14}CO_3^-$ was added to each flask, under conditions given under Table III. At the same time, ATP was added to flask b, and ATP plus Rib-5-P was added to flask c, to give a final concentration of 1.0 mM of each. After 10 min, the light was turned on and photosynthesis was allowed to proceed for another 8 min. Samples were taken at the times indicated, and total ^{14}C fixed into stable compounds in each sample was determined.[6]

RESULTS

The total fixation of $^{14}CO_2$ in Experiment 1 during light, dark, and light again is shown in Figure 1, which illustrates the sequence and number of samples taken. The rates of $^{14}CO_2$ fixation derived from the data shown in Figure 1, as well as corresponding rates from Experiments 2, 3, and 4 are shown in Table I. In each experiment the rates are high during the first light period, become zero or slightly negative in the dark, and are significantly lower during the second dark period as compared with the first. None of the additions of ATP, $NADPH_2$, Rib-5-P, Ribul-1,5-P_2, or several combinations caused any significant

dark fixation or greatly stimulated the subsequent light fixation. Some stimulation of the second light period rate by ATP (Experiments 1, 3, and 5) was seen. Increased bicarbonate concentration (Experiment 4) caused no dark fixation but may have slightly stimulated the subsequent light rate.

The levels of labeled Ribul-1,5-P_2, Fru-1,6-P_2, and Sed-1,7-P_2 in the three phases of Experiment 1 are shown in Table II. In the control, the dark level of Ribul-1,5-P_2 dropped to about one fourth its level in the first light period and then rose to a much higher level during the next light period. With added ATP and with added ATP plus Rib-5-P, the level of labeled Ribul-1,5-P_2 was maintained in the dark at about its level during the first light period. Both of these additions, but especially ATP alone, caused a considerable increase in the level of labeled Ribul-1,5-P_2 during the second light period. The levels of the other sugar diphosphates appear to have remained about constant, except for a slight drop during the dark period.

Table III gives the results of Experiment 5, in which labeled bicarbonate was added after the light was turned off, following a period of six min photosynthesis with unlabeled bicarbonate. In this case, a small dark fixation is observed, particularly during the first 3 min of darkness. The addition of ATP, and of ATP plus Rib-5-P, in flasks b and c caused about a threefold stimulation in this first three minute dark fixation rate. Even so, this fixation rate is only of the order of 2% of the rate during the subsequent light period (11-1/2 to 18 min).

Another experiment, identical with Experiment 5 except for the omission of pyrophosphate, was performed. The results, not shown, were the same in all respects as those from Experiment 5, except that all the rates were somewhat lower. The effect of added pyrophosphate on rates of photosynthesis by isolated chloroplasts has been discussed elsewhere.[9]

DISCUSSION

The results of Experiment 1, as displayed in Table II, provide clear evidence for a light activation of the carboxylation reaction. The fact that added ATP has entered the chloroplasts and has been used by enzymes of the carbon reduction cycle is indicated by the raising of the level of labeled Ribul-1,5-P_2, the carboxylation substrate, to about the same level in the dark as it was in the first light period. Subsequent light fixation shows that the carboxylation enzyme was still inactive though at a diminished activity. Thus enzyme and substrate were present in the dark, but there was no significant rate of carboxylation reaction as measured by ^{14}C incorporation into stable products. That labeled Ribul-1,5-P_2 did not rise even more with added ATP can be attributed to the using up of labeled pentose monophosphates in the chloroplasts. Addition of unlabeled Rib-5-P would not be expected to increase the pool of labeled Ribul-1,5-P_2 but might have led to the formation of more unlabeled Ribul-1,5-P_2. This increased supply of substrate would have stimulated dark fixation of $H^{14}CO_3^-$ if the enzyme for the carboxylation reaction were active in the dark.

The lowered rates of $^{14}CO_2$ in the second light period, together with the much higher levels of Ribul-1,5-P_2 during that period, clearly show that the carboxylation enzyme is never fully activated after the dark period. From previous studies[9] we know that the rate would have fallen off after 15 min from the time the chloroplasts were first brought to room temperature, even if the light were kept on and the chloroplasts were allowed to photosynthesize continuously. The results in the present study support our previous conclusion that it is principally the activity of the carboxylation enzyme that limits the rate of photosynthesis by these isolated chloroplasts after 15 min.

Heber and Santarius[10] concluded from their studies with non-aqueous isolated chloroplasts that the chloroplast membrane <u>in vivo</u> is impermeable to $NADPH_2$ and NADP. If this impermeability is also true for isolated chloroplasts, only in

the broken chloroplasts could $^{14}CO_2$ fixation in the dark be expected to be stimulated by addition of ATP and $NADPH_2$ (Experiment 2). Our chloroplast preparations usually contain 10-30% of chloroplasts that appear to have lost membrane integrity when viewed in the optical microscope with phase optics.[6] However, chloroplasts occupy only about 1% of the volume of the suspension in these experiments, thus dilution of intermediate compounds or loss of other cofactors in the non-intact chloroplasts could account for failure to observe fixation when ATP and $NADPH_2$ were supplied.

In Experiments 3 and 4, added Ribul-1,5-P_2 is as ineffective as Rib-5-P plus ATP in bringing about fixation of $H^{14}CO_3^-$ with isolated chloroplasts in the dark. These experiments were designed primarily to establish if higher levels of $H^{14}CO_3^-$ could overcome the apparent loss of carboxylation activity in the dark. Even with 0.05 M $H^{14}CO_3^-$ and added mM ATP and Rib-5-P, there was no significant fixation. The level of HCO_3^- reported[3] as necessary for obtaining half maximal velocity with the isolated enzyme is 0.01 M. It would thus appear that the light activation of the carboxylation in the intact chloroplasts is more than a lowering of enzyme affinity for HCO_3^-.

The results of Experiment 5 (Table III) show that there is some dark fixation of $^{14}CO_2$, particularly during the first minutes of darkness. This is consistent with results from *in vivo* experiments with *Chlorella pyrenoidosa*[2] and spinach chloroplasts,[4] in which the level of Ribul-1,5-P_2 falls for 1-2 min after the light is turned off and then reaches a more or less constant level.

The small dark fixation was not seen in Experiments 1 through 4 because the rate was averaged for the entire dark period. There appears to be some loss of labeled compounds that had been formed during the previous period of photosynthesis with $H^{14}CO_3^-$. This loss is probably due to conversion in the dark of some stable intermediate compounds to some unstable or volatile compounds which would be lost when the material is dried on filter paper.

From the greater dark fixation during the first 3 min, with added ATP as compared to the control, it appears that the ATP has entered the chloroplast and is stimulating the conversion of Ribul-5-P to Ribul-1,5-P_2.

Since Mg^{++} ion is the only known cofactor for the isolated, fresh and active ribulose diphosphate carboxylase,[3] it may be that the level of Mg^{++} ion is higher in the light than in the dark in the stroma region of the intact chloroplasts. The isolated enzyme is reported to have a pH optimum of about 8.[3] Conceivably pH changes occur in the chloroplasts which provide a more favorable pH in the light than in the dark. It is possible that changes in both H^+ and Mg^{++} ion operate together to provide the strong light-dark regulation evidenced by these studies.

Dilley and Vernon[11] have reported that in isolated spinach chloroplasts there is a light-dependent uptake of H^+ amounting to 0.5 to 0.7 equivalent per mole of chlorophyll, and an efflux of K^+ plus Mg^{++} ions of the same magnitude. Light activation by means of controlled levels of these ions should involve an increase in Mg^{++} at the enzyme site and an increase in pH. Thus, the reported flows of ions might appear at first to be in the wrong direction. However, the important, light-driven flow of ions seems most likely to be across the thylakoid membranes within the chloroplasts since these thylakoid membranes are the principal sites of photochemical energy conversion. In the light, this flow would result in the movement of H^+ ions from the stroma region of the chloroplasts into the thylakoids, whereas K^+ ion and Mg^{++} ion might move from the thylakoids to the stroma. Measurements of pH are made by electrodes in the suspending medium, while measurements of metal ions are made with a sample of medium from which the chloroplasts have been filtered. Thus if the chloroplasts are broken or even "leaky", the changes in ion level in the medium may reflect those changes which would be found in the stroma in intact chloroplasts. In any event, true changes in the levels of ions in the stroma

region, where the carbon cycle enzymes are thought to be located, have yet to be measured in intact chloroplasts capable of high rates of photosynthesis with CO_2.

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FIGURE CAPTIONS

Fig. 1. Effects of addition of ATP and Rib-5-P on $^{14}CO_2$ fixation by isolated spinach chloroplasts in light and dark (Experiment 1). Dark additions: x, 2×10^{-3} M NaCl; o, 1×10^{-3} M ATP, 1×10^{-3} M NaCl; ⊙, 1×10^{-3} M ATP, 1×10^{-3} M Rib-5-P.

TABLE I

Rates of $^{14}CO_2$ Uptake by Isolated Spinach Chloroplasts in Light and Dark, with Added Cofactors and Metabolites

Expt.	$NaHCO_3$ mM	Light Rate μmole ^{14}C· (mg Chl·hr)$^{-1}$	Addition mM	Dark Rate μmole ^{14}C· (mg Chl·hr)$^{-1}$	Light Rate μmole ^{14}C· (mg Chl·hr)$^{-1}$	μg Chl per flask
1.	7.5	143	Control	-3	82	63
		154	ATP	-10	104	
		145	ATP, Rib-5-P	-3	86	
2.	7.5	134	Control	-4	96	69
		139	$NADPH_2$	-5	78	
		132	$NADPH_2$, ATP	-5	71	
3.	16	139	Control	-4	107	31
		142	ATP, Rib-5-P	-3	127	
		157	Ribul-1,5-P_2	-10	90	
4.	50	115	Control	-7	72	46
		113	ATP, Rib-5-P	+1	81	
		115	Ribul-1,5-P_2	-4	86	

The rates during the two light periods are the maximum observed rate in each period. The rate during the dark period is for the total five minutes calculated on an amount per hour basis. The schedule of light and dark periods is given in Figure 1.

TABLE II

Amounts of Labeled Sugar Diphosphates Found in Chloroplast Suspensions in Light and Dark, with and without Additions

	μmoles·(mg Chl)$^{-1}$ of ^{14}C					
	Ribul-1,5-P_2			Fru-1,6-P_2 and Sed-1,7-P_2		
Vessel	a	b	c	a	b	c
After 6 min light and just before dark period	.081	.095	.083	.71	.69	.69
Dark addition (Final concen.)	2 mM NaCl	1 mM ATP 1 mM NaCl	1 mM ATP 1 mM Rib-5-P	2 mM NaCl	1 mM ATP 1 mM NaCl	1 mM ATP 1 mM Rib-5-P
After 4.75 min dark	.019	.068	.084	.61	.61	.66
3 min light following dark period	.23	.37	.27	.68	.80	.70

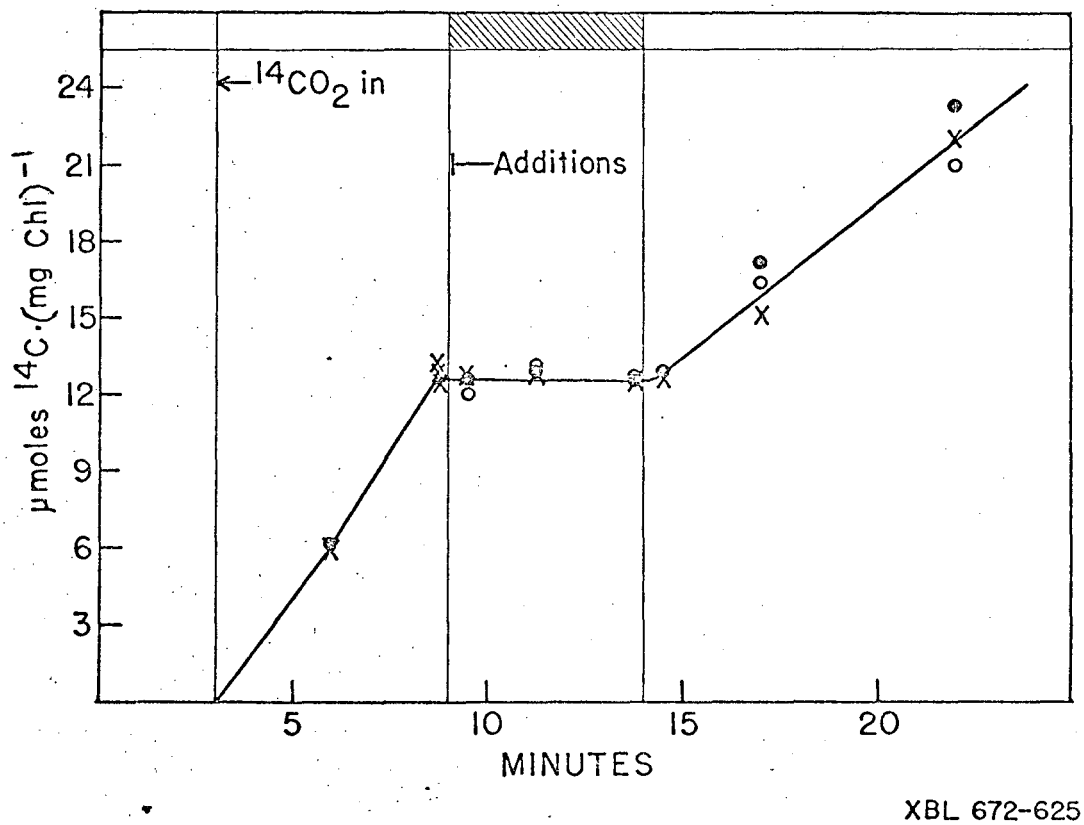
Data from Experiment 1 (see also Table I and Figure 1).

TABLE III

Rates of $^{14}CO_2$ Uptake by Isolated Spinach Chloroplasts in Light and Dark, with Added Cofactors and Metabolites (Experiment 5)

Time (min)	Total Fixed	Rate	Total Fixed	Rate	Total Fixed	Rate
0	Dark + $NaH^{14}CO_3$		Dark + $NaH^{14}CO_3$ + ATP		Dark + $NaH^{14}CO_3$ + ATP + Rib-5-P	
		1.38		2.84		2.32
3	0.069		0.142		0.116	
		0.44		1.35		1.00
9-3/4	0.119		0.294		0.228	
10	Light		Light		Light	
		56		71		75
11-1/2	1.76		2.37		2.41	
		101		118		127
18	12.74		15.20		16.12	

Each flask contained 46 μg chlorophyll in 50 μl with 1×10^{-3} M pyrophosphate in solution c.[6] Before the dark period, the chloroplasts were preilluminated for 3 min, 1.5 μmoles $H^{12}CO_3^-$ was added, and photosynthesis allowed to proceed for 6 min. Within 10 sec after the dark, 6.4 μmoles of $H^{14}CO_3^-$ (283 μC) and 0.5 μmoles of ATP and Rib-5-P were added as indicated. Total fixed is given as μmole $^{14}CO_2 \cdot (mg\ Chl)^{-1}$, while the rates (underlined) are μmoles $^{14}CO_2 \cdot (mg\ Chl \cdot hr)^{-1}$.


←$^{14}CO_2$ in
Additions
μmoles $^{14}C \cdot (mg\ Chl)^{-1}$
24
21
18
15
12
9
6
3
5
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15
20
MINUTES
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Fig. 1