

Short Communication

Effects of Intermediates of the Photosynthetic Carbon Reduction Cycle on Carbon Metabolism in Spinach Chloroplasts Illuminated on Filter Paper Discs

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Suspensions of chloroplasts isolated from leaves of spinach or peas and illuminated in suitable aqueous media are capable of both O₂ evolution and CO₂ fixation at rates comparable with those which occur *in vivo* (8, 11). Under these conditions the major radioactive products of photosynthetic ¹⁴CO₂ assimilation are glycerate-3-P and dihydroxyacetone-P, a considerable proportion of which may be recovered from the medium outside the chloroplasts (3). It is still not clear what prevents metabolism of these early products of photosynthesis to hexose phosphates and compounds such as amino acids and sucrose.

Recently we have suggested (6) that glycerate-3-P and DHAP¹ accumulate in chloroplast suspensions simply because they diffuse from the plastids into the external medium where they cannot be metabolized further. We have shown that these compounds were not accumulated if the volume of medium in the experimental sample was reduced to a minimum by layering the chloroplasts on filter paper discs. Under these conditions the chloroplasts accumulated hexose-monoP and amino acids such as glycine and alanine but did not form significant amounts of sucrose. Factors controlling the biosynthesis of sucrose from CO₂ in chloroplasts remain unknown.

It is possible that the nature of the products formed by isolated chloroplasts could depend on the relative concentrations of intermediates of the photosynthetic carbon reduction cycle within the plastids. We have therefore investigated effects of preincubating chloroplasts with varying concentrations of glycerate-3-P and sugar phosphates on the distribution of radioactivity into the products of ¹⁴CO₂ assimilation by spinach chloroplasts illuminated on filter paper discs.

Results of previous investigations by Bucke *et al.* (4), Bamberger and Gibbs (1), and Jensen and Bassham (9) indicate that the effects of added PCR cycle intermediates on rates of CO₂ fixation by chloroplasts in suspensions depend on the activity of the chloroplast preparations used. Therefore, in our investigations we have compared results obtained with chloroplasts of high photosynthetic activity with those from preparations of low activity.

METHODS

Chloroplasts were prepared from leaves of field-grown spinach (*Spinacia oleracea*) as described by Cockburn *et al.* (5), with the exception that the maximal centrifugal force used to harvest the chloroplasts was 2000g. The preparations were re-

suspended in HEPES buffered medium, pH 7.5 (6), and examined in a phase contrast microscope to determine the proportion of intact chloroplasts. Chlorophyll content was measured spectrophotometrically and the capacity of the chloroplasts to evolve O₂ was determined with a Clark-type electrode.

The chloroplasts were then diluted to a concentration of about 100 μl of chlorophyll per 100 μl of HEPES buffered medium containing glycerate-3-P, fructose-1,6-diP, fructose-6-P, or ribose-5-P at concentrations ranging from 10 μM to 10 mM and left in the dark for about 2 min. These were layered on filter paper discs and illuminated in the presence of 0.5% ¹⁴CO₂ in air as described previously (6). The chloroplasts were inactivated and the products of photosynthesis were extracted by plunging the paper discs into hot 80% (v/v) ethanol. Rates of CO₂ assimilation were calculated from the amount of radioactivity incorporated into the ethanol-soluble material. About 10% of the fixed carbon was assimilated into an ethanol-insoluble glucan (starch).

Photosynthetic products were separated by one-dimensional high voltage paper electrophoresis in pyridine-acetic acid-water (4:6:490) buffer, pH 4.5. Sugar phosphates were eluted from the electropherograms and hydrolyzed, and the products were separated by high voltage paper electrophoresis in 50 mM borate buffer, pH 9.2. The major radioactive sugar phosphates were identified as glucose-monoP, fructose-monoP, and fructose-diP. Radioactivity was also incorporated into compounds which were not intermediates of the PCR cycle. These compounds, which will be referred to as "end products," included organic acids such as malate and glycolate, amino acids such as glycine and alanine, and traces of oligosaccharides.

The distribution of radioactivity into the various products of ¹⁴CO₂ assimilation were determined directly on the electropherograms using a Geiger-Muller counter. The amount of radioactivity in a given area located by autoradiography was expressed as a percentage of the total recorded for that sample.

RESULTS

Most chloroplast preparations were capable of evolving O₂ at rates of about 60 μmoles/mg chl·hr when supplied with only water, bicarbonate, and P_i as substrates. These preparations will be termed O+ chloroplasts. A small proportion of the preparations isolated and assayed under identical conditions did not evolve O₂ unless supplied with intermediates of the PCR cycle although they contained a high proportion of intact chloroplasts. These preparations will be referred to as O- chloroplasts.

¹ Abbreviations: DHAP: dihydroxyacetone phosphate; PCR cycle: photosynthetic carbon reduction cycle.

Table I shows the results of experiments in which the chloroplasts of both types were preincubated with intermediates of the PCR cycle at a concentration of 10 mM, layered onto filter paper discs and illuminated in $^{14}\text{CO}_2$ for 10 min. In these experiments the control sample of O+ chloroplasts fixed CO_2 at a rate of about 9 $\mu\text{moles/mg chl}\cdot\text{hr}$. This is less than might have been expected on the basis of the rates of O_2 evolution, since it has been shown that isolated chloroplasts will assimilate CO_2 and evolve O_2 with a stoichiometry of one

Table I. Effects of Preincubating Spinach Chloroplasts with Intermediates of the PCR Cycle on Rates of Photosynthetic CO_2 Assimilation and on the Distribution of Radioactivity

Chloroplasts equivalent to about 60 μg of chlorophyll were layered on filter paper discs and illuminated for 10 min in 0.5% $^{14}\text{CO}_2$ in air at 25 C and 20,000 lux.

PCR Cycle Intermediate with Which Chloroplasts Were Preincubated (10 mM)	Type of Chloroplast Preparation ¹	Rate of CO_2 Fixation $\mu\text{moles/mg chl}\cdot\text{hr}$	^{14}C Recovered in Given Fraction as a Percentage of Total Recovered in Ethanol-soluble Compounds				
			Glycerate-3-P	DHAP	Sugar-diP	Sugar-monoP	End products
None	O-	0.8	3	6	5	39	47
	O+	9.0	2	10	31	24	33
Ribose-5-P	O-	3.5	3	22	5	50	20
	O+	12.5	2	5	25	48	22
Fructose-6-P	O-	2.1	2	6	8	63	21
	O+	10.0	2	10	20	55	17
Fructose-1,6-diP	O-	5.9	2	5	62	10	21
	O+	19.0	2	5	55	13	28
Glycerate-3-P	O-	2.1	3	10	45	15	27
	O+	7.2	2	5	51	13	29

¹ O+ chloroplasts were capable of high rates of O_2 evolution when illuminated with bicarbonate, H_2O and P_i alone; O- chloroplasts did not evolve O_2 unless intermediates of the PCR cycle were included in the assay medium.

to one (11). However, spectrophotometric determinations on 80% acetone extracts of replicate filter paper discs indicated that only about 60% of the applied chlorophyll was retained. Hence, it is probable that the decreased rate of CO_2 assimilation reflects the breakage of some chloroplasts during the process of layering onto the filter paper discs.

In general, preincubation with sugar phosphates increased the amount of carbon assimilated by both types of chloroplasts. However, the increase relative to the control rate was always greater in the O- chloroplasts. With both types of chloroplasts, fructose-1,6-diP produced the greatest stimulation of carbon fixation. Glycerate-3-P stimulated CO_2 assimilation by O- chloroplasts. With O+ chloroplasts this compound inhibited carbon fixation, or in some experiments had little effect.

There was a significant difference in the distribution of radioactivity into the products of photosynthesis formed by the control samples of the two types of chloroplasts. In the O- chloroplasts about 40% of the fixed carbon was incorporated into sugar-monoP and only about 5% was recovered in the sugar-diP fraction. In contrast, with the O+ chloroplasts about 30% of the fixed carbon was recovered in sugar diphosphates. Hydrolysis of the sugar diphosphates produced mainly fructose, indicating that the major sugar-diP being formed was probably fructose-1,6-diP.

When the chloroplasts were preincubated with ribose-5-P, fructose-1,6-diP or fructose-6-P, the amount of radioactivity recovered in the fraction corresponding to the fed compound increased and the amount of radioactivity recovered in products of its further metabolism decreased compared with control samples. However, results obtained when the chloroplasts were preincubated with glycerate-3-P differed. In these experiments the distribution of ^{14}C into products of photosynthesis formed by both types of chloroplasts was similar to that observed when the chloroplasts were preincubated with fructose-1,6-diP.

Both types of chloroplasts were preincubated with intermediates of the PCR cycle over a range of concentrations from 10 μM to 10 mM. The results of experiments in which fructose-1,6-diP was used are shown in Figure 1. A significant increase in the amount of carbon fixed by O- chloroplasts was observed with concentrations of fructose-diP as low as 0.1 mM

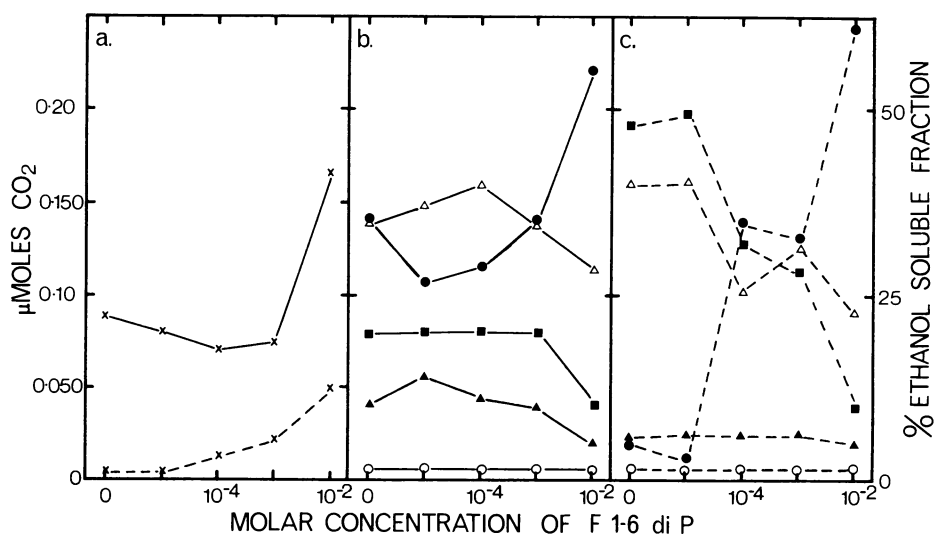


FIG. 1. Effects of preincubating spinach chloroplasts with varying concentrations of fructose-1,6-diP on (a) amounts of carbon assimilated and (b, c) the distribution of radioactivity into products of photosynthesis. Solid lines: O+ chloroplasts; broken lines: O- chloroplasts. Fractions: O—O: glycerate-3-P; ▲—▲: dihydroxyacetone-P; ■—■: sugar monophosphates; ●—●: sugar diphosphates; △—△: "end products" (mainly malate, glycolate, glycine, and alanine). Chloroplasts equivalent to about 60 μg chlorophyll were layered on filter paper discs and illuminated in 0.5% CO_2 in air at 25 C and 20,000 lux for 10 min.

(Fig. 1a). With O+ chloroplasts such an increase was only observed at the highest concentration of fructose-diP used. Changes in the distribution of radioactivity among the products of photosynthesis were observed at the same concentrations (Fig. 1, b and c).

Results of experiments carried out with other intermediates of the PCR cycle were basically similar. With O+ chloroplasts glycerate-3-P, ribose-5-P, and fructose-6-P had little effect on either the amount of carbon fixed or the distribution of ^{14}C into products unless used at a concentration of 10 mM. In contrast, fixation of CO_2 by O- chloroplasts was stimulated by concentrations as low as 0.1 mM ribose-5-P and fructose-6-P and by 1 mM glycerate-3-P. Changes in the distribution of radioactivity into products of photosynthesis were observed with 10 μM ribose-5-P, fructose-6-P, 0.1 mM fructose-diP, and 1 mM glycerate-3-P.

DISCUSSION

The changes observed in the present experiments in amounts of carbon assimilated by chloroplasts which had been preincubated with intermediates of the PCR cycle were basically similar to those reported from experiments on chloroplasts illuminated in suspensions (1, 4, 9). However, the present results differ from those of previous investigations (1, 2) in that the chloroplasts preincubated with PCR cycle intermediates at concentrations sufficient to alter their rates of CO_2 assimilation showed pronounced changes in the distribution of radioactivity between products of photosynthesis.

With added ribose-5-P, fructose-diP, and fructose-6-P the observed changes were as might be expected from isotope dilution effects if a relatively large nonradioactive pool of the given intermediate had been introduced into the chloroplast. That is, the amount of ^{14}C recovered in the fraction corresponding to the fed compound increased, and the amount of radioactivity recovered in products of its further metabolism decreased.

These isotope dilution effects will only be seen when the amount of exogenous substrate assimilated by the chloroplasts is large compared with both the endogenous pool and the amount being formed by assimilation of $^{14}\text{CO}_2$. The rate of incorporation of isotope into the fed intermediate must also be high compared with the rate of its further metabolism. Hence, these experiments can provide information on (a) the penetration of compounds into the chloroplast, (b) the relative size of endogenous pools of intermediates of the PCR cycle, and (c) the relative rates of metabolism of carbon through various reactions of the PCR cycle.

The present results indicate that all the PCR intermediates investigated can be rapidly assimilated by the chloroplasts. They also indicate that the endogenous concentrations of PCR cycle intermediates in the O- chloroplasts were much lower than those in the O+ chloroplasts. However, the low activity of these chloroplasts cannot be due to the low level of PCR cycle intermediates alone, since in this case incubation with such compounds should restore their photosynthetic activity completely. The O- chloroplasts would appear to be similar to the chloroplasts isolated in a salt-based medium by Plaut and Gibbs (10).

With both types of chloroplast preparations the greatest stimulation of CO_2 fixation was observed in those samples which had been preincubated with fructose-1,6-diP. Fructose-6-P and ribose-5-P were less effective although closer to the metabolic pathway to the CO_2 acceptor ribulose-1,5-diP. The observed isotope dilution effects indicate that these compounds had penetrated the chloroplasts, but they also indicate that they were being metabolized slowly compared with the rate of CO_2 fixation. Hence, it is unlikely that the stimulation observed

with fructose-diP resulted from replenishing of the pool of ribulose-1,5-diP. However, these results are consistent with previous suggestions (2, 9) that the level of fructose-diP within the chloroplasts could affect the rate of CO_2 assimilation, possibly by a feedback mechanism. In this case the activity of fructose-1,6-diphosphatase could control both the rate of entry of carbon into the PCR cycle and the rate of loss of carbon through hexose-monoP. In the present experiments the accumulation of radioactivity in the sugar-monoP fraction by the control sample of O- chloroplasts suggests that in these plastids this control might have been lost. This would account both for the low levels of endogenous PCR cycle intermediates and for the low levels of photosynthesis in these chloroplasts.

Everson *et al.* (7) have suggested that the relative activities of the enzymes involved in the conversion of triose-P to hexose-monoP could control the nature of products formed by isolated chloroplasts. It is postulated that with chloroplasts in which diphosphatase activity is much less than fructose 1,6-diP aldolase, carbon would tend to accumulate in compounds in equilibrium with triose-P, while high diphosphatase activity would favor formation of fructose-6-P, which should favor sucrose synthesis. However, our present results indicate that even under conditions favoring accumulation of hexose-monoP sucrose may not be formed in significant amounts.

In the present experiments little difference was observed in the distribution of radioactivity into products of photosynthesis in chloroplasts which had been preincubated with glycerate-3-P and in those which had been preincubated with fructose-diP. This suggests that both types of chloroplasts were capable of rapidly converting glycerate-3-P to fructose-diP. For the observed isotope dilution effects to occur, the rate at which this conversion occurred must have been considerably faster than both the rate of CO_2 fixation and the rate of hydrolysis of fructose-diP. Hence, it would appear that in these chloroplasts sucrose synthesis was limited by one of the reactions associated with the further metabolism of hexose-monoP.

These results also support our previous suggestion that chloroplast suspensions accumulate early intermediates of the PCR cycle merely because they leak from the chloroplasts into the external medium.

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