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Short Communication

Starch Degradation in Isolated Spinach Chloroplasts[1]

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ABSTRACT

A method for loading isolated intact spinach (*Spinacia oleracea* L.) chloroplasts with ^{14}C-starch is described. These intact chloroplasts were incubated aerobically in the dark for 30 minutes. Radioactivity in starch declined and glyceric acid 3-phosphate and maltose were the major radioactive products. It is proposed that starch is degraded within the chloroplast to glyceric acid 3-phosphate and to maltose.

Recent advances (1) in chloroplast metabolism indicate that carbon is removed from the photosynthetic carbon reduction cycle primarily as glycolate, triose-P, glycerate-3-P, and starch. The first three compounds, in contrast to the insoluble polysaccharide, move freely from the chloroplast to the cytoplasm where they undergo further metabolism. While the subsequent metabolism of these soluble compounds has been the subject of exhaustive studies, little attention has been given to the mobilization of this major nutritional polysaccharide. An analysis of the recent literature would indicate that the glucan is broken down within the organelle to triose-P prior to export, since glucose and hexose mono- and diphosphates are transported to a limited extent across the chloroplast membrane (2, 6). Supporting evidence for a glycolytic pathway within the organelle is the finding of a fructose-6-P kinase located within the chloroplast (3). In this paper, we report preliminary experiments on the dark metabolism of ^{14}C-starch in a preparation of isolated chloroplasts. To perform these experiments, spinach chloroplasts with isotope predominately in starch were prepared by taking advantage of the impermeability of the polysaccharide to the chloroplast envelope, in contrast to other compounds which become labeled during a short period of photosynthesis in $^{14}CO_2$.

MATERIALS AND METHODS

Preparation of Chloroplasts. Chilled, field-grown spinach (*Spinacia oleracea* L.) leaves (10-15 g) were cut into 40 ml of 0.33 M sorbitol, 50 mM HEPES (pH 6.8), 2 mM EDTA, and 1 mM each of $Na_4P_2O_7$, $MgCl_2$, and $MnCl_2$. The mixture was agitated for 2 sec in a VirTis 45 homogenizer. The homogenate was passed through Miracloth and centrifuged at 5 C for 1.5 min at 2500*g*. The pellet was washed in 5 ml of grinding solution and centrifuged for 45 sec at 2500*g*. The pellet was finally suspended in the grinding solution with 50 mM HEPES, pH 7.8 replacing the buffer at pH 6.8.

Assimilation of $^{14}CO_2$ and Enhanced Retention of Isotope in Insoluble Fraction of Chloroplast. Chloroplasts were allowed to photosynthesize in a flat sided "lollipop" vessel (50 ml) illuminated from both sides with 3000 lux provided by 150-w incandescent lamps. Temperature was maintained at 24 C by passing the light through a lucite container filled with chilled H_2O. The chloroplasts were kept in suspension by introducing a stream of air into the reaction mixture. After a period of photosynthesis, the contents of the "lollipop" were emptied in the dark into a centrifuge tube. Aliquots were removed for the determination of the rate and products of photosynthesis. Soluble products were washed from the remainder of the chloroplast suspension by diluting with chilled 0.33 M sorbitol, 50 mM HEPES (pH 7.8), 2 mM EDTA, and 1 mM each $Na_4P_2O_7$, $MgCl_2$, and $MnCl_2$. This was followed by centrifugation for 1.5 min at 2500*g*. The washed pellet was then resuspended in the same buffer to the original chloroplast density as described in the table and figure legends.

Products of Fixation. Radioactivity assimilated into acid-stable products was determined by mixing a 0.4-ml sample of the reaction mixture with 25 μl of 90% formic acid and placing an aliquot on a lens paper-covered planchet. Radioactivity was measured with a Nuclear-Chicago gas flow counter.

Radioactive compounds were separated by applying aliquots of the reaction mixture following treatment with formic acid to Whatman No. 1 paper with subsequent chromatography in Woods GW_3 medium (7) or *n*-butyl alcohol-pyridine-H_2O (6:4:3). The phosphorylated compounds were identified by treatment with alkaline phosphatase followed by rechromatography of the reaction mixtures with authentic compounds. Maltose was confirmed by co-chromatography of the radioactive substance eluted from the chromatography paper and of the glucose produced as the result of hydrolysis in 1 N HCl with standards. In this report, the material remaining at the origin of the chromatogram is termed starch. Identification as a glucan was accomplished by reacting this material with 1 N HCl for 60 min in a tube placed in a boiling H_2O bath. The HCl was removed by evaporation, and the reaction mixture was chromatographed. Glucose was the only radioactive product.

RESULTS AND DISCUSSION

Table I records the retention of the radioactive products in the reaction mixtures before and after a wash in isotonic buffer. Washing removed over 90% of the radioactivity, resulting in starch representing approximately half of the remaining isotope. In agreement with others (2, 6; M. J. Harvey and M. Gibbs, unpublished), the phosphorylated sugars were retained by the chloroplasts. Maltose behaved similarly to the starch.

Figure 1 illustrates that the glucan and fructose-1,6-diP were degraded in the dark by the chloroplasts which had been treated as described in Table I. The major products were glycerate-3-P, maltose, and to a lesser extent, glucose-P (a mixture of glucose-1-P and glucose-6-P).

In another experiment (Fig. 2), disalicylpropanediamine, which has been shown by Robinson *et al.* (4) to increase radioac-

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Table I. *Distribution of Radioactivity in Products*

A: Chloroplasts (0.20 mg Chl/ml) were photosynthesized in air for 30 min in a total volume of 16 ml containing 0.33 M sorbitol, 50 mM HEPES (pH 7.8), 2 mM EDTA, 1 mM each of $Na_4P_2O_7$, $MgCl_2$, $MnCl_2$, fructose-1,6-diP, and 4.35 mM $NaH^{14}CO_3$, 2 μCi/μmol. Photosynthesis was terminated by emptying the reaction mixture into a darkened centrifuge tube. One aliquot was assayed for total radioactivity and another one was subjected to paper chromatography. B: To 7.5 ml of the reaction mixture of A were added 16 ml of chilled 0.33 M sorbitol, 50 mM HEPES (pH 7.8), 2 mM EDTA, and 1 mM each of $Na_4P_2O_7$, $MgCl_2$, and $MnCl_2$, followed by centrifugation for 1.5 min at 2500*g*. The pellet was resuspended in 7.5 ml of the same buffer and portions were assayed for total radioactivity and for distribution of isotope. All washing operations were carried out at 0 to 5 C. The time from the end of illumination to the sampling of the resuspended pellet was 7 min.

	Total Radioactivity	Radioactivity in					
		Glycerate-3-P	Fructose-1,6-diP	Glucose-P	Maltose	Starch	Others
	cpm/mg Chl $\times 10^{-6}$	*% of total*					
A. Before washing	3.39	51	23	5	3	9	6
B. After washing	0.22	9	21	7	5	52	2

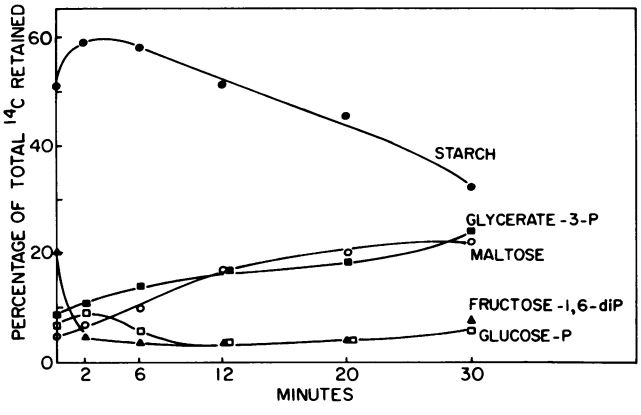

FIG. 1. Time course of dark metabolism of products formed during photosynthesis. Two ml of the suspension containing 0.20 mg Chl/ml washed chloroplasts (see Table IB) were incubated under air together with 1 mM potassium phosphate, pH 8.8, and 50 μM phenazine methosulfate in darkened tubes at 20 C. Samples were removed, and products were identified by paper chromatography. The total radioactivity retained in the washed chloroplasts was 2.2×10^5 cpm/mg Chl.

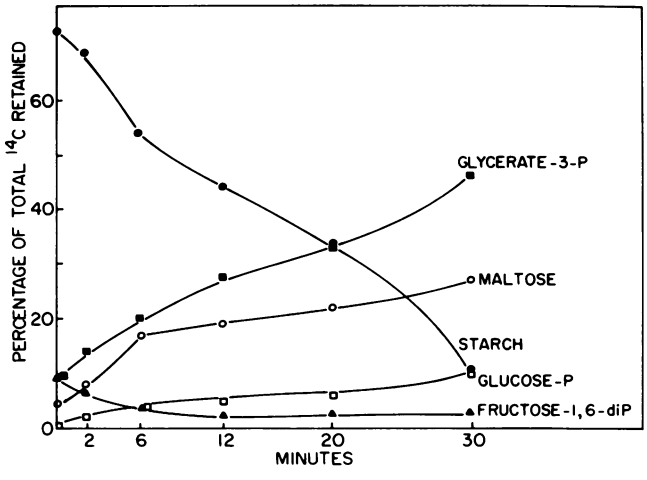

FIG. 2. Time course of dark metabolism of products formed during photosynthesis in the presence of disalicylpropanediamine and phenazine methosulfate. Chloroplasts containing 0.33 mg Chl/ml were allowed to photosynthesize in 11 ml of the medium described in Table I with the inclusion of 100 μM disalicylpropanediamine. After 25 min of photosynthesis, 50 μM phenazine methosulfate was added. Five min thereafter photosynthesis was terminated. The chloroplasts were washed as described in Table I, except that 11 ml of the chilled buffer were added to 6.5 ml of chloroplasts, which were then centrifuged and resuspended to a total of 6.5 ml. The time from the end of illumination to the sampling of the resuspended pellet was 5 min. Two-ml aliquots of the resuspended chloroplasts, containing 0.33 mg Chl/ml (1.6×10^5 cpm/mg Chl) were incubated in the dark as described in Fig. 1.

tivity in starch during photosynthesis in $^{14}CO_2$ by spinach chloroplasts, was added to the reaction mixture. In contrast to the experiment described in Figure 1, phenazine methosulfate was added to the photosynthesizing chloroplasts 5 min prior to termination of the light phase. Under these conditions, radioactivity was further enhanced in the starch and movement of isotope into the products during the subsequent dark phase was more rapid than that depicted in Figure 1. This effect of phenazine methosulfate was not resolved.

Unpublished experiments in this laboratory (M. J. Harvey and M. Gibbs) had demonstrated that triose-P can be oxidized within the chloroplast to glycerate-3-P. In this report, we show that starch formed in the chloroplast during photosynthesis was also metabolized to glycerate-3-P. The degradative pathway appeared to be Embden-Meyerhof since radioactivity was not detected in gluconate-6-P or ribulose-5-P.

Maltose has recently been reported as a product of photosynthesis in spinach chloroplasts (5). Furthermore, maltose phosphorylase was detected in extracts from young spinach leaves and proposed to be the enzyme responsible for the synthesis of the disaccharide from glucose-1-P and glucose (5). In our preparations, maltose appears to be a degradative product of starch since labeled glucose was not detected. However, it is possible that there was sufficient unlabeled glucose within the chloroplast to complement glucose-1-P derived from the photosynthetically labeled starch. On the other hand, it appears quite reasonable that maltose phosphorylase may catalyze the synthetic reaction while an amylolytic reaction functions in the reverse direction.

The presence of a degradative glycolytic pathway in the chloroplast raises a number of intriguing questions. Pertinent are the source of ATP required in the fructose-6-P kinase reaction and the nature of the reaction catalyzing the reoxidation of reduced pyridine nucleotide resulting from the dehydrogenation of glyceraldehyde-3-P. The ATP may well be substrate derived from glyceraldehyde-3-P dehydrogenase coupled to glycerate-3-P kinase and malic acid dehydrogenase substituting for phenazine methosulfate employed in these experiments could complete the dismutation reaction. Both enzymes have been demonstrated in the chloroplast and their substrates are known to move freely across the plastid envelope (2).

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