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Characterization of Starch Breakdown in the Intact Spinach Chloroplast[1]

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ABSTRACT

Starch degradation with a rate of 1 to 2 microgram-atom carbon per milligram chlorophyll per hour was monitored in the isolated intact spinach (*Spinacia oleracea*) chloroplast which had been preloaded with ^{14}C-starch photosynthetically from $^{14}CO_2$. Starch breakdown was dependent upon inorganic phosphate and the ^{14}C-labeled intermediates formed were principally those of the Embden-Meyerhof pathway from glucose phosphate to glycerate 3-phosphate. In addition, isotope was found in ribose 5-phosphate and in maltose and glucose. The appearance of isotope in the intermediates of the Embden-Meyerhof pathway but not in the free sugars was dependent upon the inorganic phosphate concentration. Dithiothreitol shifted the flow of ^{14}C from triose-phosphate to glycerate 3-phosphate. Iodoacetic acid inhibited starch breakdown and caused an accumulation of triose-phosphate. This inhibition of starch breakdown was overcome by ATP. The inhibitory effect of ionophore A 23187 on starch breakdown was reversed by the addition of magnesium ions. The formation of maltose but not glucose was impaired by the ionophore. The inhibition of starch breakdown by glycerate 3-phosphate was overcome by inorganic phosphate. Fructose 1,6-bisphosphate and ribose 5-phosphate did not affect the rate of polysaccharide metabolism but increased the flow of isotope into maltose. Starch breakdown was unaffected by the uncoupler (trifluoromethoxyphenylhydrazone), electron transport inhibitors (rotenone, cyanide, salicylhydroxamic acid), or anaerobiosis. Hexokinase and the dehydrogenases of glucose 6-phosphate and gluconate 6-phosphate were detected in the chloroplast preparations. It was concluded (*a*) that chloroplastic starch was degraded principally by the Embden-Meyerhof pathway and by a pathway involving amylolytic cleavage; (*b*) ATP required in the Embden-Meyerhof pathway is generated by substrate phosphorylation in the oxidation of glyceraldehyde 3-phosphate to glycerate 3-phosphate; and (*c*) the oxidative pentose phosphate pathway is the probable source of ribose 5-phosphate.

In 1862, Sachs (14) demonstrated that carbon assimilated during photosynthesis by the chloroplast accumulated in the form of starch. The synthesis of this polyglucan from well characterized intermediates of the photosynthetic carbon reduction cycle has been amply documented. In recent publications from this laboratory, preliminary evidence was presented indicating that in the intact spinach chloroplast starch synthesis and degradation were regulated by the level of Pi (17) and that starch breakdown occurred by a pathway similar to the Embden-Meyerhof pathway (10). In addition to the phosphorylated compounds of glycolysis, maltose and glucose were also observed. Therefore, the conclusion was reached that the glucosidic bonds in the polysaccharide were cleaved either by Pi or water. We report here additional experiments on the regulation of starch breakdown in the isolated spinach chloroplast.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* var. Long Standing Bloomsdales) was grown according to Peavey and Gibbs (12). Isolated intact chloroplasts were prepared by the method of Gibbs and Robinson (5). Fully expanded leaves (10–15 g) were homogenized for 2 sec in 50 ml of chilled isolation medium (50 mM HEPES-NaOH [pH 6.8], 330 mM sorbitol, 10 mM Na_2EDTA, 2 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, and 1 mM DTT). The chloroplasts were pelleted at 750*g* for 1 min. To load the isolated chloroplasts with ^{14}C-starch, the photosynthetic reaction medium contained 50 mM HEPES-NaOH (pH 7.8), 330 mM sorbitol, 2 mM Na_2EDTA, 2 mM $MgCl_2$, 0.25 mM NaH_2PO_4, 1 mM DTT, and 3.5 to 8.85 mM $NaH^{14}CO_3$ with a specific radioactivity of 7 to 15 mCi/mmol. The photosynthetic conditions were 22.5 C, 440 w/m^2, and 50 to 150 μg Chl/ml. Chl was determined by the procedure of Arnon (2). After a photosynthetic period of 10 to 15 min, 10 ml of chloroplast suspension was washed with 30 ml chilled reaction buffer containing 50 mM HEPES-NaOH (pH 7.8), 330 mM sorbitol, 2 mM Na_2EDTA, 2 mM $MgCl_2$, and 5 mM NaH_2PO_4 (except where noted). This portion of the protocol is termed "the wash." The chloroplasts were pelleted at 750*g* for 1 min. The chloroplast fraction was resuspended in 1 to 2.5 ml of reaction buffer with changes noted in the figure and table legends. Starch breakdown was monitored for 30 to 60 min in the dark.

During starch degradation and photosynthesis, 0.5 ml aliquots were removed at intervals, and the reaction was stopped with 50 μl of concentrated formic acid. Total ^{14}C incorporation into acid-stable intermediates was determined with a Nuclear-Chicago gas flow end window detector system. ^{14}C-Labeled starch was determined in two ways. The sample was filtered through a MF-Millipore membrane filter (0.45 μm pore size) and washed several times with 95% ethanol. Of the isotopically labeled compounds, only polyglucan remained on the filter. In a second procedure, the ^{14}C-labeled starch together with the other labeled intermediates was separated by one-dimensional paper chromatography in the GW_3 solvent (20). Hydrolysis (1 N HCl at 100 C) of the starch fraction obtained either by filtration or by chromatography yielded only labeled glucose. The results obtained by both methods did not differ more than 5%. The identity of maltose was confirmed by co-chromatography and hydrolysis (either in 1 N HCl at 100 C or by glucosidase, Sigma G-7256, pH 7.5 at 25 C). The phosphorylated intermediates

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were phosphatased and rechromatographed against known free sugars in GW_3 and "semi-stench" (7).

RESULTS AND DISCUSSION

Chloroplasts containing photosynthetically labeled starch were used to characterize starch degradation which took place at a rate of 1 to 2 μg-atom carbon/mg Chl·hr. After a photosynthetic period of 10 to 15 min, followed by a buffer wash, pelleting, and resuspension in the medium with buffer containing 2.5 to 5 mM Pi, about 40 to 80% of the radioactivity was located in starch with lesser amounts distributed among triose-P (essentially dihydroxyacetone-P), glycerate-3-P, ribose-5-P, fructose-6-P, glucose-6-P, fructose-1,6-BP,[4] glucose, and maltose. Most likely this distribution of radioactivity was the result of retention during the washing period and from starch breakdown between the termination of photosynthesis and resuspension in buffer, a period of approximately 5 min.

As monitored by $^{14}CO_2$ photoassimilation, less than 1% of the chloroplasts were ruptured during washing, pelleting, and resuspension. Starch disappearance continued linearly up to 1 hr. Preparations which were osmotically shocked had rates of starch breakdown less than 1% of the intact plastid.

Time Course of Starch Breakdown. The degradation products of starch breakdown are shown in Figure 1. Roughly 20% of the radioactivity in the starch was removed linearly in 30 min. Concomitant to the decrease in the level of radioactivity in starch and hexose mono- and bisphosphates, were increases in ribose-5-P, triose-P, glycerate-3-P, maltose, and glucose. The ratio of glucose and fructose phosphates to fructose-1,6-BP tended to be approximately 1.5:1 while the ratio of glycerate-3-P to triose-P was about 2:1. The two free sugars, glucose and maltose, accounted for considerable portions (up to 15%) of the radioactivity after 30 min.

Phosphorolytic Pathway of Starch Degradation. The formation of triose-P and glycerate-3-P suggested the Embden-Meyerhof pathway while the presence of ribose-5-P was indicative of the oxidative pentose-P pathway functioning in the degradation of starch. The following experiments were carried out to determine the role of each pathway.

Glyceraldehyde-3-P dehydrogenase and glucose-6-P dehydrogenase are key enzymes of glycolysis and of the oxidative pentose-P pathway, respectively. DTT is known to activate the former but inactivate the latter enzyme (1). On addition of 10 mM DTT, the rate of starch breakdown was unaffected, whereas the pool of glycerate-3-P was increased 61% mainly at the expense of triose-P, and ribose-5-P fell off by 44% (Table I). These inverse changes in the levels of glycerate-3-P and ribose-5-P could be the result of enhancing the oxidation of glyceraldehyde-3-P while blocking the oxidative pentose-P pathway at its initial enzyme. Since the rate of starch breakdown was unaffected, the rate-limiting step must lie elsewhere.

Interestingly, DTT caused a decrease in glucose concomitant with an increase in glucose-6-P. This observation is consistent with finding of hexokinase in our preparations with a rate of 0.5 to 1 μmol glucose esterified/mg Chl·hr. Hexokinase is known to be activated by sulfhydryl reagents (16).

Data suggestive of an anaerobic glycolysis came from experiments with iodoacetate, the well known inhibitor of glyceraldehyde-3-P dehydrogenase and with inhibitors and uncouplers of electron transport. Inasmuch as the incubations were carried out in darkened tubes, it became quite clear that a mechanism other than the photochemical act must be present in the chloroplast to produce ATP required in the fructose-6-P kinase reaction. A mitochondrial pathway for ATP generation was eliminated by observing that uncouplers of phosphorylation such as NH_4Cl (0.1–10 mM) or 0.1 to 10 mM trifluoromethoxyphenylhydrazone, nitrogen gas, and electron transport inhibitors such as rotenone, cyanide, or salicylhydroxamic acid had no effect on starch breakdown. The latter two inhibitors were without effect when presented singly or together in order to eliminate both Cyt *c* oxidase and the alternative pathways (18). We conclude from these results that ATP arises by substrate phosphorylation during the oxidation of glyceraldehyde-3-P to glycerate-3-P.

Further evidence for this conclusion is supported by the results obtained with iodoacetic acid. If starch breakdown occurs via anaerobic glycolysis, then inhibition of glyceraldehyde-3-P dehydrogenase should lead to an accumulation of triose-P and a concomitant inhibition of starch breakdown due to a lack of ATP. The addition of iodoacetate resulted in a 40 and 50% inhibition of starch breakdown at concentrations of 10 and 25 mM, respectively, with a striking increase in triose-P (Fig. 2). The amylolytic breakdown of starch was unaffected. Since adenosine nucleotides penetrate the intact spinach chloroplast at a rate comparable to starch breakdown in our preparations (9), we tested their effect on starch breakdown in the presence and absence of iodoacetate. In the absence of inhibitor, various concentrations (0.1–20 mM) of ATP, ADP, or AMP did not alter the rate of starch breakdown. On the other hand, 10 mM ATP overcame the sulfhydryl inhibitor and restored starch breakdown with an accumulation of triose-P (Fig. 2).

To determine the presence and contributions of the oxidative

Table I. Effect of dithiothreitol on starch breakdown

The concentration of dithiothreitol was 10mM. At 0 time, the starch fraction in the control contained 34000 cpm or 69.4% of the total radioactivity within the chloroplasts. The incubation period was 30 min. The Δ% in the Tables and Figures was calculasted as: [%^{14}C(zero min)]-[%^{14}C(30 min)].

	Control			Dithiothreitol		
	0 min	30 min		0 min	30 min	
	%^{14}C		Δ%	%^{14}C		Δ%
Starch	69.4	45.9	-23.5	67.6	44.4	-23.2
Maltose	6.3	9.0	+ 2.7	6.4	9.3	+ 2.9
Glucose	1.4	5.7	+ 4.3	1.4	6.7	+ 5.3
Glucose-6-P	7.3	5.2	- 2.1	9.1	7.1	- 2.0
Fructose-1,6-BP	5.1	3.8	- 1.3	4.3	3.6	- 0.7
Fructose-6-P	2.1	1.8	- 0.3	2.4	2.8	+ 0.4
Ribose-5-P	1.3	7.4	+ 6.1	1.4	7.1	+ 5.7
Triose-P	2.4	7.5	+ 5.1	1.4	1.6	+ 0.2
Glycerate-3-P	4.7	13.7	+ 9.0	6.0	17.4	+11.4

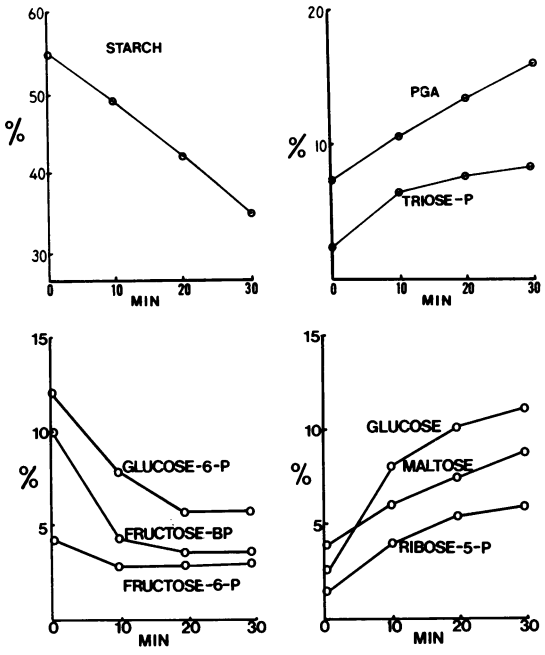

FIG. 1. Time course of the ^{14}C products of starch degradation in the presence of 5 mM inorganic phosphate. Each determination represented the average of 10 experiments.

[4] Abbreviation: BP: bisphosphate.

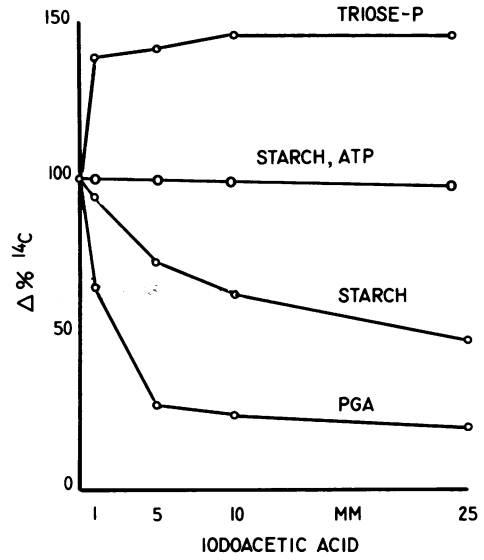

FIG. 2. Effect of iodoacetate and ATP on starch breakdown. At 0 time the starch fraction of the control represented 20,000 cpm and 24% was degraded during the 30-min incubation. The concentration of ATP was 10 mM. These values represented three experiments.

pentose-P pathway, enzymes of the pathway were determined and liberation of $^{14}CO_2$ was monitored. Glucose-6-P and gluconate-6-P dehydrogenases reported earlier by others (8, 15) were found in our preparations with specific activities of 1 to 2 and 0.48 to 0.88 μmol substrate consumed/mg Chl·hr. On the other hand, $^{14}CO_2$ was not detected in a 30-min incubation nor was gluconate-6-P seen on our chromatograms. Experiments in the absence of DTT or in the presence of 50 μM to 5 mM phenazine methosulfate did not result in the evoluation of $^{14}CO_2$. Phenazine methosulfate at 500 μM inhibited starch breakdown by 50%. The lack of isotopically labeled CO_2 is not conclusive since the level of radioactivity in carbon atom 1 of the glucosyl moiety in the polysaccharide was not determined.

Hydrolytic Degradation of Starch. Clearly, the increasing levels of maltose and glucose during incubation were indicative of an amylolytic cleavage of the labeled polysaccharide (Table I and Fig. 1). The derivation of glucose by glucose 6-phosphatase does not appear to be viable since this enzyme has not been reported in chloroplast preparations and there is no evidence that DTT inhibits phosphatase activity. Other properties of the amylolytic pathway will be dealth with elsewhere.

Effect of Mg^{2+} and A 23187 on Starch Breakdown. The ionophore A 23187 has been shown to catalyze a H^+/Mg^{2+} and H^+/Ca^{2+} exchange across the chloroplast membrane (13). Since Mg^{2+} is required for a number of enzymes such as phosphorylase and fructose-6-P kinase catalyzing the conversion of starch to glycerate-3-P and α-amylase utilizes Ca^{2+} as a stabilizer (6), it would be expected that the ionophore would influence the time course and the products of starch degradation by the phosphorolytic and hydrolytic pathways. The results are recorded in Table II.

The addition of 20 μM ionophore inhibited starch breakdown about 50% and increased fructose-1,6-BP by 5-fold. The sharp decrease in the rate of starch breakdown was expected since the enzyme initiating phosphorolytic cleavage is magnesium-dependent. The accumulation of fructose-1,6-BP at the expense of the 3-carbon compounds may be explained by an inhibition of the magnesium-requiring PGA kinase resulting in an excess of triose phosphates. However, the equilibrium of the aldolase reaction strongly favors the conversion of triose phosphates to hexose diphosphate.

With respect to the free sugars, the ionophore essentially eliminated [^{14}C]glucose but had little effect on the appearance of labeled maltose. The disaccharide is generally thought to be derived immediately from starch by β-amylolytic activity while there are conflicting reports concerning the pathway of glucose formation (11). The data for maltose recorded in Table II were consistent with the finding that β-amylase does not require Ca^{2+} (6). Regardless of the derivation of glucose, it would appear that the monosaccharide was not liberated exclusively from maltose.

Finally, it should be noted that the addition of 5 mM Mg^{2+} to a preparation challenged by 20 μM ionophore restored starch breakdown. In contrast, 5 mM Mg^{2+} in the absence of A 23187 did not affect starch breakdown. This observation contrasts with the report (3) that similar concentrations of Mg^{2+} inhibited CO_2 fixation by the spinach chloroplast.

Regulation of Starch Degradation by Phosphorylated Intermediates. The "phosphate translocator" is responsible for the transport of glycerate-3-P and Pi across the inner membrane of the chloroplast (19). Glycerate-3-P not only competitively inhibits the uptake of Pi but will also cause the efflux of chloroplastic Pi. The addition of glycerate-3-P to the incubating medium should be a means of resolving the phosphorolytic and hydrolytic pathways.

As shown in Table III, inhibition of starch breakdown was achieved by the addition to the reaction buffer of an amount of glycerate-3-P equivalent to Pi in the wash buffer. The reverse addition restored to some extent starch breakdown. Decreased concentrations of Pi whether by omission from the buffers or by addition of glycerate-3-P tended to limit the appearance of isotope in glycerate-3-P. This effect could be the result of a need of Pi for the substrate phosphorylation generated in the glycerate-3-P kinase reaction. A lack of ATP would result in accumulation of hexose monophosphate coupled to a less rapid decrease in fructose-1,6-BP. This condition was observed only when starch breakdown was most severely inhibited. In all likelihood, Pi plays a regulatory role directly or indirectly in many steps of the glycolytic pathway. We conclude from the data presented here that a principal site of Pi regulation was phosphorylase. This conclusion is consistent with the reported rather high K*m* of 4 to 7.5 for plant phosphorylase (4, 6).

Finally, it should be noted that the appearance of isotope in

Table II. Effect of ionophore A23187 and magnesium on starch breakdown

The concentration of ionophore A23187 and magnesium were 20μM and 5 mM, respectively. At 0 time, the starch fraction represented 35,750 cpm or 65% of the radioactivity within the chloroplasts. The incubation period was 30 min.

	$-Mg^{2+}$ −A23187	$+Mg^{2+}$ −A23187	$-Mg^{2+}$ +A23187	$+Mg^{2+}$ +A23187
		Δ% ^{14}C		
Starch	-21.5	-21.9	-8.7	-21.8
Maltose	+ 3.0	+ 3.3	+3.4	+ 3.6
Glucose	+ 7.7	+ 6.0	+0.9	+ 7.2
Glucose-6-P	- 5.3	- 4.3	-4.8	- 3.9
Fructose-1,6-BP	- 2.8	- 2.0	+0.7	- 3.1
Fructose-6-P	- 1.2	- 0.3	-0.9	- 0.9
Ribose-5-P	+ 7.1	+ 5.7	+5.0	+ 6.5
Triose-P	+ 5.4	+ 5.0	+2.5	+ 3.5
Glycerate-3-P	+ 7.6	+ 8.5	+1.9	+ 8.9

Table III. Effect of inorganic phosphate and glycerate-3-P on starch breakdown

Phosphate and glycerate-3-P were 5 mM. "Wash" represented the 30 ml chilled reaction buffer used to wash the pellet and resuspended the chloroplasts after photosynthesis. "Reaction" is the reaction buffer in which starch breakdown was monitored after 30 min. At 0 time, the starch fraction represented 54.3% of a total of 60000 cpm within the chloroplast. These values were obtained from the average of five experiments.

Wash;	+Pi	-Pi	+PGA	+Pi
Reaction;	+Pi	-Pi	+Pi	+PGA
		Δ% ^{14}C		
Starch	-15.0	-6.8	-14.0	-9.9
Maltose	+ 4.1	+4.4	+ 3.7	+3.1
Glucose	+ 5.8	+3.6	+ 3.6	+4.0
Glucose-6-P	-10.1	-7.6	- 6.0	-6.3
Fructose-1,6-BP	- 8.5	-6.7	- 4.3	-5.6
Fructose-6-P	+ 3.9	+7.0	+ 1.8	+1.1
Ribose-5-P	+ 3.0	+0.7	+ 2.0	+2.5
Triose-P	+ 4.8	+2.0	+ 1.4	+3.8
Glycerate-3-P	+12.0	+3.4	+11.8	+7.3

maltose and glucose was unaffected by the level of Pi. Indeed, when starch breakdown was limited by Pi, maltose and glucose represented the major products of starch breakdown.

It has been established that intermediates of the reductive pentose-P cycle, when added to photosynthesizing chloroplasts, have considerable effects on the assimilation of CO_2. Therefore, we performed experiments to determine if these compounds could regulate starch breakdown. The presence of 1, 10, or 20 mM fructose-1,6-BP or ribose-5-P did not influence the rate of starch degradation. On the other hand, as shown in Table IV, fructose-1,6-BP and ribose-5-P altered the glycerate-3-P to triose-P ratio, decreased label in ribose-5-P, and increased label in maltose. The altered ratio was most likely due to isotopic dilution of the triose-P pool by unlabeled carbon resulting in an apparent inhibition of the oxidation of triose-P to glycerate-3-P. That the pentose-P responded similarly to the hexose di-P was apparently the result of both compounds entering the stroma as triose-P rather than the parent compound. It has been demonstrated that the inner membrane of the chloroplast is slowly permeable to ribose-5-P and less so to fructose-1,6-BP (9). In addition, due to chloroplast breakage, these preparations contain the enzymes in the reaction medium necessary to convert fructose-1,6-BP and ribose-5-P to triose-P (data not presented). The decrease of isotope in ribose-5-P could be caused by an inhibition of the oxidative pentose-P pathway but most probably by the isotopically diluted triose-P pool since part of the ribose-5-P could be the result of a transketolase-reaction involving fructose-6-P and glyceraldehyde-3-P.

Most interesting was the increased isotopic labeling of maltose when starch breakdown took place in the presence of an added sugar phosphate. In some experiments, after a 30-min incubation, maltose accounted for 35% of the ^{14}C label derived from starch. An explanation must await further characterization of the enzymes catalyzing the pathway resulting in the formation of free sugars.

Distribution of Products between Chloroplast and Supernatant Fraction. When the plastids were spun down after starch breakdown, of the phosphorylated intermediates, glycerate-3-P and triose-P to a large extent were located in the supernatant fraction (Table V). Smaller amounts of ribose-5-P and fructose-1,6-BP were also found in this fraction. While starch and maltose remained in the chloroplast, about 8% of the glucose was found in the supernatant solution. This level of glucose outside the plastid was unexpected since we found that starch remained essentially unlabeled after incubating the plastids with [^{14}C]glucose under photosynthetic conditions.

Table IV. Effect of fructose-1,6-bisphosphate and ribose-5-phosphate on starch breakdown

The experiments were performed in the standard reaction mixture containing 10mM fructose-1,6-BP or 10mM ribose-5-P. At 0 time the starch fraction represented 19,350 cpm of a total radioactivity of 45,000 cpm. The incubation period was 30 min.

	Control	Fructose-1,6-bisphosphate	Control	Ribose-5-phosphate
		Δ% ^{14}C		
Starch	-13.8	-14.0	-13.6	-13.8
Maltose	+ 2.0	+ 4.9	+ 1.6	+ 4.5
Glucose	+ 8.9	+ 6.7	+10.4	+10.2
Glucose-6-P	-15.3	-14.6	-12.8	-14.8
Fructose-1,6-BP	-10.5	-10.6	- 9.2	- 7.4
Fructose-6-P	- 3.2	- 3.9	- 3.7	- 3.7
Ribose-5-P	+ 9.1	+ 3.2	+ 7.7	+ 3.5
Triose-P	+ 7.8	+23.1	+ 7.8	+17.8
PGA	+15.0	+ 5.2	+11.9	+ 3.7

Table V. The distribution of the ^{14}C intermediates of starch breakdown inside and outside the chloroplast

After 30 minutes of starch breakdown, the chloroplasts were centrifuged at 750 g for 60 seconds. The supernatant was decanted. The ^{14}C labeled intermediates were determined as in Materials and Methods.

	Total ^{14}C	Pellets	Supernatant
	%	% of total ^{14}C	
Starch	39.1	98.8	1.2
Maltose	8.7	99.6	0.4
Glucose	7.4	91.8	8.2
Glucose-6-P	8.3	99.1	0.9
Fructose-1,6-BP	6.2	97.4	2.6
Fructose-6-P	4.1	98.2	1.8
Ribose-5-P	6.1	91.3	8.7
Triose-P	8.9	58.1	41.9
Glycerate-3-P	11.2	65.7	34.3

CONCLUSIONS

Our results confirm and extend earlier reports from this laboratory (10, 17) that starch located within the intact spinach chloroplast was degraded by the Embden-Meyerhof pathway and also by a pathway involving amylolytic cleavage. The concentration of Pi among other factors appeared to regulate the formation of phosphorylated compounds or free sugars. While the enzymes of the oxidative pentose-P pathway were present, control of glucose-6-P metabolism by that pathway or the Embden-Meyerhof pathway requires further experimentation. An anaerobic glycolytic pathway requires a dismutation reaction for the turnover of reduced pyridine nucleotide. In bacteria, this role is usually assumed by alcohol and lactate dehydrogenases. Since these enzymes are lacking in leaves, another means must be sought.

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