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Physiological Rates of Starch Breakdown in Isolated Intact Spinach Chloroplasts[1]

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MARK STITT AND HANS W. HELDT
Lehrstuhl fuer Biochemie der Pflanze, Untere Karspuele 2, 3400 Goettingen, Federal Republic of Germany

ABSTRACT

Starch breakdown with rates above 10 μatom carbon per mg chlorophyll per hour has been monitored in spinach chloroplasts and compares favorably with the rates in whole leaves. Intact starch-loaded chloroplasts were prepared from protoplasts to avoid rupture during mechanical homogenization and rapid centrifugation. Particular attention was paid to the identification of all the products of starch degradation and to measuring the actual rates of their accumulation. The products of starch breakdown included triose phosphate, 3-phosphoglycerate, CO_2, glucose, and some maltose. Comparison of the rates of metabolism of added glucose and of the conversion of starch to phosphorylated intermediates showed that starch phosphorolysis was the major pathway leading to phosphorylated endproducts. From the results, the relative contribution of phosphorolysis and hydrolysis to starch breakdown and the contribution of glycolysis and the oxidative pentose phosphate cycle can be estimated. Phosphate has a large influence on the metabolism of the chloroplast in the dark.

Photosynthetic carbon fixation occurs in the stroma. Most of the fixed carbon is then exported as triose-P (12) into the cytosol, where it is used for sucrose synthesis (17). A significant proportion of the photosynthate can be temporarily retained in the chloroplast as starch, especially when Pi is low (3, 20), Starch accumulates in the light and is broken down in the subsequent dark period. The questions arise as to how the starch is degraded, in what form the products are exported from the chloroplast, and to what extent the starch is used in chloroplast energy metabolism. In pea chloroplasts, the breakdown is predominantly phosphorolytic (11, 22, 24), and the HMP[2] is further metabolized by glycolysis and the OPP cycle to triose-P and PGA (21, 22), which can be exported by the phosphate translocator (2). Some maltose is also formed (11, 22). We concluded that chloroplast metabolism of starch could make a significant contribution to the carbohydrate oxidation of the whole leaf (21–23). As pea leaves contain relatively low levels of starch, it seemed important to study starch breakdown in leaves with a high starch content, such as spinach.

During starch breakdown in spinach chloroplasts, formation of glucose, some maltose, triose-P, and PGA has been found (3, 10, 15). However, as the measured rates of starch breakdown were at least one order below the likely physiological rates, it is not possible to evaluate the physiological significance of these results. By paying attention to the procedures for isolation of chloroplasts and for measurement of starch breakdown, we have been able to demonstrate physiological rates of starch breakdown in isolated, starch-loaded spinach chloroplasts. From the results, it is possible to distinguish the contributions to spinach chloroplast metabolism of phosphorolytic and hydrolytic starch breakdown and of glycolysis and the OPP pathway.

METHODS

Spinach (*Spinacia oleracea* L., U. S. Hybrid 424, Ferry-Morse Seed Co., Mountain View, CA) was grown in water culture (13) under a 10-h-light, 14-h-dark cycle. Lighting was provided by 100 w neon tubes (1/0.7 m^2) supplemented with 100 w radium lamps (1/0,24 m^2; Osram, Munich), and the temperature was 24 to 27 C in the light and 15 to 20 C in the dark. Leaves were preilluminated for 8 to 20 h to obtain a high starch content.

For measurement of starch degradation in whole leaves, leaf discs (combined fresh weight 0.25 to 0.35 g) were taken from a pair of matched leaves, combined, and immediately killed by boiling in 80% (v/v) alcohol. At each time point, separate samples were prepared from five different pairs of leaves. All the leaves were attached to the plant and held in a box lined with damp filter paper. Starch was determined as in Stitt *et al.* (24), except that the glucose was determined by enzymic analysis (25).

Protoplasts were prepared from spinach palisade cells, as previously described (25), but the Metrizamide (Sigma) density gradient was replaced by a Percoll (Pharmacia, Uppsala, Sweden) density gradient which contained 0.5 M sorbitol, 5 mM Mes (pH 6.0), 1 mM $CaCl_2$, throughout, with 20% (v/v) Percoll and unpurified protoplasts in phase III (4.6 ml), 16% (v/v) Percoll in phase II (2 ml), and no Percoll in phase I (2 ml). After centrifugation (250*g*, 7 min), protoplasts collected at the interface between phases I and II.

To prelabel starch, protoplasts (100 to 150 μg Chl·ml^{-1}, were illuminated (180 × 10^3 ergs $cm^{-2}s^{-1}$) in an O_2 electrode in 0.5 M sorbitol, 5 mM Mes (pH 6.0), 0.5 mM [^{14}C]HCO_3 (25 to 35 Ci·mol^{-1}) until O_2 evolution ceased (7 to 9 min) because all the bicarbonate had been fixed. The protoplasts were centrifuged (5 min, 250*g*); resuspended in 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA; passed three times through a 17-μm nylon net; and layered over a discontinuous Percoll density gradient. The Percoll had been dialyzed for 24 h in 50-ml portions against three changes of 1 liter distilled H_2O before use. Undialyzed Percoll strongly inhibited the photosynthesis of free chloroplasts. The gradient contained 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM EDTA, throughout. Under the protoplast extract, were layers of 2 ml 18% (v/v) Percoll and 4 ml 22% (v/v) Percoll. The gradient was centrifuged for 10 min at 950*g*, and the chloroplasts were recovered from the sediment. In some experiments, a 2-ml cushion of 50% (v/v) Percoll was also included so that the chloroplasts layered on the 22%:50% interface.

The resulting chloroplasts (20 to 30 μg Chl·ml^{-1}) were resuspended in 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 1 mM $MnCl_2$, 1

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[2] Abbreviations: HMP, hexosemonophosphate; OPP, oxidative pentose phosphate; PGA, 3 phosphoglycerate; FBP, fructose 1.6-bisphosphate; SBP, sedoheptulose 1.7-bisphosphate; RuBP, ribulose 1.5-bisphosphate.

mM $MgCl_2$, 2 mM EDTA (for other additions, see text) and incubated in the dark at 20 C in 70-μl aliquots in 1.5-ml Eppendorf microcentrifuge tubes. CO_2 was collected in 50 μl 10% (w/w) KOH contained in the cut tip of a 400 μl microfuge tube held on the wall of the 1.5-ml tube by silicone grease. The incubation was terminated by adding 10 μl 60% $HClO_4$. For each treatment, at least three replicate incubations were carried out. Radioactivity in starch and soluble compounds was separated by centrifugation (3). The soluble radioactivity was separated into anionic, cationic, and neutral components by ion exchange resins (25). Neutral compounds were further analyzed by TLC (25), and anionic compounds were analyzed by ^{14}C anion exchange chromatography (Wirtz, unpublished), based on the procedure described for ^{32}P-labeled extracts (12).

For enzymic analysis of substrate levels, chloroplasts were isolated by a Percoll gradient, as above, incubated (50 to 100 μg Chl/ml^{-1}) for 30 min, quenched with 5% (v/v) $HClO_4$, and neutralized 15 min later with 5 M KOH/1 M triethanolamine, and substrates were measured as previously described (25). For analysis of [^{14}C]glucose metabolism, 0.5 mM or 2 mM [U-^{14}C]glucose (14 Ci·mol^{-1}) or [1-^{14}C]-, [2-^{14}C]-, or [6-^{14}C]glucose (8 Ci·mol^{-1}) were added to 70 μl chloroplasts (100 μg Chl·ml^{-1}) and incubated and analyzed for [^{14}C]CO_2, and radioactive labeling of different compounds was done as described above. Acid-volatile radioactive impurities were removed from isotopes before use. Each experiment included at least 3 replicate incubations. Chl was measured and glucose in the chloroplast compartment of protoplasts was determined as in Stitt *et al.* (25).

RESULTS AND DISCUSSION

Starch Breakdown in Leaves. Figure 1 shows the decrease in starch content of leaves on the plant during the dark period. The spinach leaves were identical to those used for the preparation of chloroplasts. After darkening, starch degradation starts without appreciable lag and continues linearly until almost all of the starch has been degraded, with an average rate of 12 μatom carbon/mg Chl·h during the first 12 h of darkness.

Preparation of Starch-Loaded Intact Chloroplasts. Good rates of starch breakdown require intact chloroplasts containing ample starch. An accumulation of starch in chloroplasts is achieved by preillumination of leaves for several hours. Conventional mechanical methods of chloroplast isolation, which normally yield good intact chloroplasts from spinach, yield poor preparations from such starch-loaded leaves. Under phase contrast, many broken chloroplasts can be seen, and the majority of the starch grains are free in the medium. The large starch grains presumably destroy the envelope. Therefore, we used protoplasts as a starting material for chloroplast isolation.

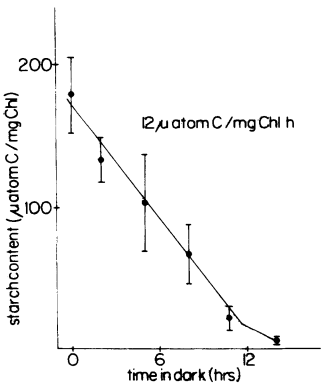

FIG. 1. Starch breakdown in spinach leaves. Each *point* represents mean ± SE of five separate samples. Leaf discs were taken from two separate matched leaves for each sample.

The chloroplasts still retained most of their starch when they were isolated from the protoplasts on an isoosmotic Percoll gradient. Under phase contrast, they appeared highly intact, and all contained two to six white starch grains. Few or no starch grains were visible outside the chloroplasts. In a typical gradient (Fig. 2), the intact chloroplast band contained 81% of the applied Chl and 62% of the applied starch. This coincidence was not fortuitous, as a completely different distribution of starch was found when lysed chloroplasts were applied to the gradient. The starch content of the chloroplasts, 86 μatom carbon/mg Chl, is representative of the amounts found in spinach leaves carrying out rapid starch breakdown (Fig. 1).

Measurement of Starch Breakdown. The most direct way to measure starch breakdown in chloroplasts would be to measure the decrease in starch during an incubation. Unfortunately, this method cannot be directly applied, since less than 4% of the starch would be degraded within a time of 30 min, and such a difference cannot be accurately measured. Therefore, starch was prelabeled with ^{14}C during 10 min-photosynthesis, and the redistribution of radioactivity out of the starch into products was followed in a subsequent dark period. The newly synthesized labeled starch is preferentially degraded (10, 20). By establishing a quantitative and corresponding increase in ^{14}C in the products, it can be ensured that all the major products have been identified. However, on its own, this approach does not provide a reliable estimate of the rate of starch breakdown, as a considerable degradation of unlabeled starch occurs simultaneously, leading to a large and unknown isotope dilution. To calculate the absolute rates, enzymic substrate analysis is used to measure how rapidly the identified products accumulate.

Since CO_2 could be one of the products of starch breakdown, it was essential to prepare ^{14}C-labeled chloroplasts which were free from [^{14}C]CO_2. Repeated centrifugation could not be used to wash the chloroplasts because it damaged them. Starch was prelabeled by supplying a low concentration of [^{14}C]bicarbonate to protoplasts at pH 6. In these conditions, a high CO_2 concentration is in equilibrium with a low bicarbonate concentration. Photosynthesis was allowed to continue until essentially all of the radioactivity was fixed, and [^{14}C]CO_2-free chloroplasts were then prepared with minimal centrifugation, avoiding breakage of the fragile organelles. When chloroplasts were kept in the dark, radioactivity in starch decreased (Fig. 3), and an equivalent increase was found in the products. At the various times, the increase in ^{14}C in CO_2 and soluble products represented 90 to 110% of the ^{14}C lost from starch. About 10% of the radioactivity appeared in CO_2, and the

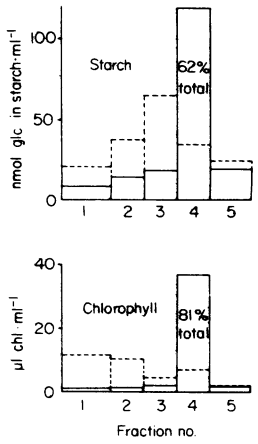

FIG. 2. Presence of starch in the chloroplasts isolated from protoplasts using an isoosmotic Percoll density gradient. *Top*, starch; *bottom*, Chl. Gradient with intact (——) and broken (- - -) chloroplasts. Total starch in the chloroplasts was 86 μatom Carbon/mg Chl.

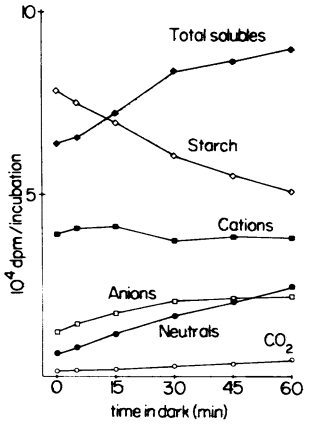

FIG. 3. Breakdown of ^{14}C labeled starch in spinach protoplasts. Protoplasts were prelabeled with [^{14}C]CO_2 and passed three times through a nylon net, and the resulting chloroplasts were isolated by an isoosmotic Percoll gradient, incubated in 5 mM Pi in the dark, and quenched in $HClO_4$ after various time periods, CO_2 driven off after acidification was adsorbed in KOH. The insolubles were then separated by centrifugation, and the resulting supernatant (combined with CO_2 to give total solubles) was separated into anions, cations, and neutrals using ion exchange columns.

Table I. *Calculation of the Rate of Starch Breakdown in Starch-Loaded Spinach Chloroplasts*

Protoplasts from starch-loaded leaves were used to isolate chloroplasts, either directly or after prelabeling with [^{14}C]HCO_3. In seven separate experiments, the redistribution of ^{14}C out of starch into glucose, maltose, anions, and CO_2 was determined (Fig. 3), providing an estimate of the relative fluxes from starch into these products. In four experiments with unlabeled chloroplasts, the rate of accumulation of glucose and of HMP, fructose bisP, triose-P, and PGA (combined as anions) was measured by enzymic substrate analysis (Table II). The rates of CO_2 and maltose formation were extrapolated from the measured rate of glucose accumulation and the relative distribution of ^{14}C between glucose, CO_2, and maltose.

	Increase in Labeling as Percentage of ^{14}C Lost from Starch	Rate of Accumulation	
		Measured by enzymic analysis	Extrapolated
		μmol/mg Chl·h	
Total Products	106		10.4
Anions	38 ± 12	4.6 ± 2.8	
CO_2	9 ± 2		0.8
Neutral	59 ± 13		
Glucose	39 ± 10	3.3 ± 0.7	
Maltose	19 ± 5		1.7

rest was equally distributed between anionic and neutral compounds. No increase was found in cationic compounds, although they represented a major portion of the total label present initially in the chloroplasts. Glucose accounted for most of the increase of ^{14}C in neutral compounds, with a smaller contribution from maltose (Table I). With respect to the anions, radioactivity rose mainly in PGA and triose-P (Fig. 4). The radioactivity in HMP decreased. No starch breakdown was observed when lysed chloroplasts were incubated (experiments not shown).

The changes in the levels of various intermediates, measured by enzymic analysis after a 30-min incubation of the chloroplasts with 5 mM Pi, are shown in Table II. The difference between the initial and the final values reveals a marked accumulation of the

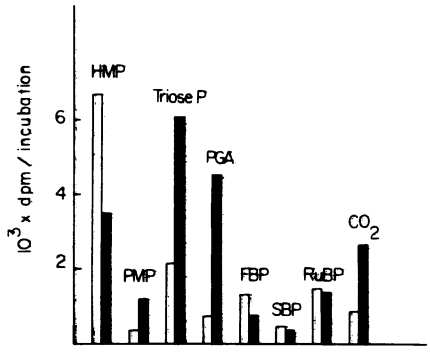

FIG. 4. Change in ^{14}C in various phosphorylated intermediates and CO_2 during starch mobilization with 5 mM Pi. Phosphorylated intermediates were separated by ionexchange chromatography. Radioactivity present is given after 0 (unhatched) and 30 (hatched) min.

Table II. *Accumulation of Products of Starch Mobilization as Measured by Enzymic Substrate Analysis*

Chloroplasts were prepared from protoplasts, using a Percoll gradient, and incubated (50 μg Chl·ml^{-1}) with 5 mM Pi. Samples were quenched with 5% $HClO_4$. The samples were neutralized exactly 15 min later.

Substrate	Amount after Incubation with Pi		Rate of Accumulation
	0 min	30 min	
	nmol/mg Chl		*μatom C/mg Chl·h*
Glucose 6-P	151	173	0.26
Fructose 6-P	46	56	0.12
Fructose 1,6-bisP	11	12	0.01
Triose-P	97	361	1.58
PGA	22	493	2.83
Glucose	361	1175	9.76
Fructose	293	715	5.06
Sucrose	987	423	−10.45
(Glucose − Fructose)			4.70
(Glucose − 0.5 ΔSucrose)			4.54

anionic compounds, with the major products being PGA and triose-P. The position is more complex for the neutral compounds. In our experience, preparations of spinach chloroplasts, whether isolated from protoplasts or by conventional mechanical methods, contain sucrose or related oligosaccharides, which do not seem to derive from a cytoplasmic contamination (Stitt, unpublished data) and which are hydrolyzed to glucose and fructose during incubation in the dark (Table II). However, glucose increases far more than can be accounted for by such hydrolysis. The increase in glucose (9.8 μatom·mg Chl) is greater than the increase in fructose (4.7) or the decrease of glucose in sucrose (4.5). This additional accumulation of glucose was attributed to starch breakdown, since glucose had already been shown to be a major product of ^{14}C starch degradation (Table II). In the following experiments, the fructose accumulation was always measured, and an equivalent amount of glucose was subtracted from the total glucose to calculate the rate at which glucose was formed from starch. The rate of formation of the minor products, CO_2 and maltose, could not be measured accurately.

Rates and Pathways of Starch Breakdown. Table I summarizes seven separate analyses of ^{14}C-starch breakdown and four separate enzymic analyses of substrate accumulation. During 30 min in the dark with 5 mM Pi, glucose was formed at 3.3 μatom carbon/mg Chl·h. The anionic compounds, representing PGA, triose-P, FBP, and HMP, increased at 4.6 μatom carbon/mg Chl·h. From the

absolute rates of glucose and of anion formation and from the relative distribution of ^{14}C between the various endproducts, rates of 0.8 and 1.7 μatom carbon/mg Chl·h can be extrapolated for CO_2 evolution and maltose formation, respectively. Summing the individual rates for all the products yields a total rate of 10.4 μmol carbon/mg Chl·h, comparing favorably with the rate found in leaves (Fig. 1). The rate could not be increased by adding ATP, varying the pH, or changing the Pi concentration. After separating the chloroplasts and medium by silicone oil centrifugation, the phosphorylated intermediates, and also glucose and maltose, were almost exclusively found in the medium (experiment not shown). Phosphorylated intermediates can be exported by the phosphate translocator (2) and glucose and maltose by the glucose translocator (18) (Herold, unpublished).

It seems, from our data, that starch can be used in two ways. It can be hydrolytically degraded into free sugars, which are released from the chloroplast. Spinach chloroplasts contain hydrolytic enzymes (1, 16) which could catalyse maltose formation, although the exact origin of the glucose is not clear since maltase is apparently absent (14) (Stitt, unpublished). Alternatively, the immediate products of starch degradation are retained inside the chloroplast and metabolized as far as CO_2 triose-P, and 3PGA, before being released from the chloroplast. Such metabolism can provide energy for the chloroplast. The first step in the conversion will involve converting starch to HMP. This could occur either *via* the starch phosphorylase in spinach chloroplasts (14, 19) or *via* a hexokinase acting on glucose which is initially released from starch by a hydrolytic attack.

Metabolism of Glucose in Spinach. If phosphorylation of glucose by hexokinase is a major route, then these spinach chloroplasts should phosphorylate supplied glucose at a rate comparable to the rate at which starch is converted into CO_2 and phosphorylated intermediates. For a rigorous test, this glucose should be supplied at a concentration similar to that found in chloroplasts during starch breakdown. To determine the glucose concentration in the stroma, chloroplasts were separated from the medium by silicone oil centrifugation (2). In three separate preparations, 0.75 to 2 mM glucose was found in the stroma during starch degradation. This is likely to be a small overestimate, because sucrose was present in the acid-quenched samples, and some acid hydrolysis of the sucrose was unavoidable. Similar levels of glucose were found *in situ* in chloroplasts from starch-loaded protoplasts.

Intact chloroplasts converted 2 mM [U-^{14}C]glucose into anionic compounds, CO_2 and starch (Fig. 5). Similar observations have been made in pea chloroplasts (22). This metabolism of glucose was not due to cytosolic contamination, and it occurred within the chloroplasts (Table III), as the release of [^{14}C]CO_2 was prevented when chloroplasts were lysed by osmotic shock or addition of Triton X-100. Although glucose 6-P is a direct intermediate between glucose and CO_2, it produced a smaller isotope dilution than did more distant intermediates such as PGA, triose-P, and ribose 5-P, because the glucose 6-P is not transported across the envelope (2). Addition of ATP or ADP did not increase [^{14}C]CO_2 evolution. ATP did increase ^{14}C labeling of anions, but this represented merely an increase in formation of HMP outside the chloroplast, probably due to a hexokinase located outside the chloroplast, as in pea (24).

The overall rate of esterification of 2 mM [U-^{14}C]glucose in isolated starch-loaded spinach chloroplasts was 0.55 μatom carbon/mg Chl·h (Fig. 5). It is unlikely that the rate of glucose uptake is limiting in these experiments, as published data (18) reveal a transport rate of 13 to 30 μatom carbon/mg Chl·h with 2 mM glucose. Since the rate of glucose phosphorylation is one order of magnitude lower than the rate of anion and CO_2 production during starch degradation (5.5 μatom carbon/mg Chl·h), it is probable that HMP is mainly produced by phosphorolysis during starch breakdown, with the phosphorylation of glucose

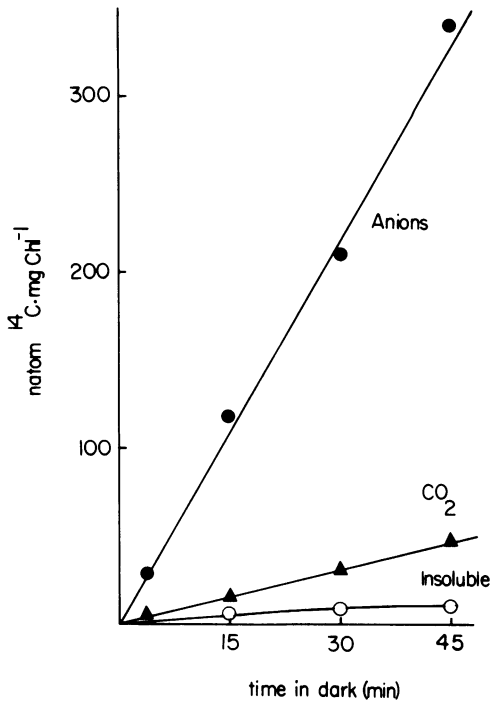

FIG. 5. Metabolism of 2 mM [U-^{14}C]glucose by starch-loaded intact spinach chloroplasts. Starch-loaded chloroplasts were prepared (by using a Percoll gradient) from protoplasts and incubated at 100 μg Chl/ml in 5 mM Pi with 2 mM [U-^{14}C]glucose in the dark. Label in anionic compounds (●—●), CO_2 (▲—▲), and insolubles (○—○) was determined. The results are the mean of three separate samples.

Table III. *[U-^{14}C]Glucose-Dependent [^{14}C]CO_2 Evolution by Intact Spinach Chloroplasts Carrying Out Starch Breakdown*

Starch-loaded chloroplasts were prepared from protoplasts using a Percoll gradient and incubated (100 μg Chl·ml^{-1}) in the dark for 30 min in the presence of 2 mM [U^{14}C]glucose (14 μCi/μmol) and added substance, as indicated.

Pi	Other Additions	[^{14}C]CO_2 Evolution
mM		*nmol/mg Chl·h*
Nil		89
Nil	Dihydroxyacetone-P, 0.5 mM	22
Nil	Ribose 5-P, 0.5 mM	23
Nil	3PGA, 0.5 mM	24
Nil	Glucose 6-P, 0.5 mM	44
Nil	Triton X-100, 0.5%	6
10		64
10	ATP, 0.5 mM	71
10	ADP, 0.5 mM	64
10		4[a]

[a] The sample was osmotically shocked.

making only a minor contribution. It should be mentioned that the rate of phosphorylation increases with glucose concentration, and, if higher concentrations ever occurred physiologically, the phosphorylation of glucose could play a more important role. The rate of glucose metabolism is comparable to that found in chloroplasts from pea shoots (22). Especially when starch is absent, glucose might serve as substrate for maintaining at least a minimal rate of chloroplast metabolism in the dark. This conclusion is not at variance with previous observations that illuminated spinach chloroplasts incorporated [^{14}C]glucose into starch at negligible rates compared with those obtained from [^{14}C]bicarbonate (3), because rates of metabolism are generally far higher in the light

than in the dark.

Evidence for Operation of the OPP Pathway in Spinach Chloroplasts. The question then arises as to how the HMP is converted to triose-P and, ultimately, to PGA. This could occur directly, *via* stromal phosphofructokinase (8), or the OPP pathway could make a contribution. The release of CO_2 during both glucose (Fig. 5) and starch metabolism (Table I) indicated that the OPP pathway was involved. We checked in two ways that this CO_2 was released by operation of the OPP pathway inside chloroplasts. First, various intermediates were added, and their effect on $[^{14}C]CO_2$ release during starch mobilization was determined (Table IV). Ribose 5-P and triose-P produce a marked isotope dilution of $[^{14}C]CO_2$ release. These compounds can enter the chloroplast, and they are intermediates in the OPP cycle. On the other hand, glucose 6-P and 6P-gluconate produce little change in $[^{14}C]CO_2$ release. Although these metabolites are intermediates in the OPP cycle, they produce no isotope dilution because they are not transported across the envelope. Compounds which enter the chloroplast (2) but are not intermediates in the OPP cycle (like 3 PGA, P-enolpyruvate, malate, and aspartate) have little effect on the release of $[^{14}C]CO_2$. The alterations in $[^{14}C]CO_2$ produced by these various intermediates involved isotope dilution rather than an inhibition of starch degradation, since total starch mobilization was only slightly affected by the various intermediates (Table IV). Second, independent evidence that the CO_2 is released from the OPP cycle in chloroplasts is provided by the preferential release of $[^{14}C]CO_2$ from [1-^{14}C]glucose (compared to [2-^{14}C]glucose or [6-^{14}C]glucose) which is diagnostic for the OPP cycle (7). This release is not due to cytosolic contamination, because $[^{14}C]CO_2$ release during glucose metabolism was prevented by lysis but was not inhibited by adding intermediates like glucose 6-P and 6 P-gluconate (Table V). An earlier study failed to find OPP activity in spinach chloroplasts (13), but this can probably be ascribed to the insensitivity of the methods used.

Carbon Cycling in the Chloroplast OPP Pathway. As usually defined (7), the OPP cycle converts 3 HMP to 3 pentose-P and 3 CO_2 and then recycles the pentose-P, forming one molecule of triose-P and two molecules of hexose-P. Overall, a HMP molecule would then be converted to 3 CO_2 and 1 triose-P molecule. Based on work in a reconstituted spinach stroma system (4), it has been suggested that, in chloroplasts, the OPP pathway operates only as far as pentose-P, which is then exported, rather than undergoing further interconversion to triose-P and HMP. In this case, the OPP pathway could not contribute to the net synthesis of triose-P from starch. In our intact chloroplasts, there is strong evidence that pentose-P is recycled. First, the release of $[^{14}C]CO_2$ from [2-^{14}C]glucose can occur only after a recycling of pentose-P (7). Second, during degradation of uniformly labeled starch, more radioactivity is released as CO_2 than accumulates as pentose-P (Fig. 4). A conversion of uniformly labeled glucose residues from starch into CO_2 and pentose-P only would yield 5 times more ^{14}C radioactivity in pentose-P than in CO_2. Thus, our data clearly demonstrate that the pentose-P is further metabolized in the chloroplasts. The effects of dilution may explain why pentose-P was not further metabolized in a reconstituted system.

Table IV. *Effect of Various Intermediates on the Release of $[^{14}C]CO_2$ during ^{14}C Starch Degradation*

Chloroplasts were prelabeled, as in Figure 3, and incubated for 30 min in the dark in the presence of the added substances (0.5 mM). The label in CO_2 and starch was determined at the beginning and end of this period, and the change is presented. Each result is the mean of three separate incubations.

	CO_2 Formation		Starch Degradation	
	nil Pi	Pi, 5 mM	nil Pi	Pi, 5 mM
	10^2 dpm/incubation			
Nil	18	10	112	113
Ribose 5-P	4	5	80	103
Triose-P	3	3	94	95
3PGA	12	14	81	101
2PGA	14	11	105	96
PEP	15	11	103	107
Pyruvate	19	11	121	109
Malate	20	11	108	102
Aspartate	20	11	111	103
Glucose 6-P	17	8	111	103
Gluconate-6-P	18	10	106	105

Table V. *$[^{14}C]CO_2$ Release from Specifically Labeled Glucose Supplied to Intact Starch-Loaded Spinach Chloroplasts*

Chloroplasts were isolated and incubated, as in Figure 4, for 30 min, with 0.5 mM [1-^{14}C]glucose, [2-^{14}C]glucose, or [6-^{14}C]glucose and Pi, if indicated. The results are the means of 2 separate experiments, each with 3 replicate samples.

Glucose Labeled in Carbon	CO_2 Release	
	Pi, 5 mM	nil Pi
	nmol CO_2/mg Chl·h	
1	10.4	8.5
2	2.4	2.6
6	1.9	4.9

According to classic formulation of the OPP cycle (7), it is not possible to release either carbons 4-6 of HMP or the carbons of triose-P as CO_2. This is because a triose-P molecule, or the basal phosphorylated end of a sugar P molecule, can only react as an acceptor in the transaldolase or transketolase reactions and are reincorporated into the basal carbons of the resulting sugar monophosphate. However, a distinct and significant release of $[^{14}C]CO_2$ from [6-^{14}C]glucose is seen with spinach chloroplasts (Table V). This can even exceed the release of $[^{14}C]CO_2$ from [2-^{14}C]glucose and can be up to half the release of $[^{14}C]CO_2$ from [1-^{14}C]glucose. Similar observations were made with pea chloroplasts (22). Two explanations for this release of $[^{14}C]CO_2$ from [6-^{14}C]glucose can be suggested. One is a recycling of triose-P, involving the hydrolysis of a C-1 phosphate of a sugar bisphosphate (22). Radioactivity is exchanged between corresponding positions of dihydroxyacetone P and glyceraldehyde 3-P by triose-P isomerase. These compounds then resynthesize HMP *via* aldolase and fructosebisphosphatase or sedoheptulose bisphosphatase. The carbons 6-4 of the original HMP will then be 50% exchanged into the positions 1-3 of the newly formed HMP and can subsequently be released as CO_2 in the OPP pathway. This requires fructosebisphosphatase or sedoheptulosebisphosphatase activities in the stroma in the dark as high as 0.5 μmol hexose/mg Chl·h. It is, at present, not clear whether such activities occur. An alternative explanation is that some of the rearrangement of pentose-P to HMP and triose-P in chloroplasts occurs *via* a mechanism resembling the L-path of OPP pathway, described recently for liver (26). The L-path differs from the orthodox, or F-, path, in that it involves arabinose 5-P, sedoheptulose 1,7-bisP, octulose 1,8-bisP and octulose 8-P as intermediates. It includes aldolase and a newly characterized phosphotransferase as enzymes, leading to a randomization of the label from [6-^{14}C]glucose into the C-1 position of the rearranged HMP, which will subsequently be released as CO_2 when the HMP is cycled around the OPP pathway.

Contribution of the OPP Cycle and Glycolysis. These results show that, in the presence of 5 mM phosphate, both the OPP cycle and glycolysis make a significant contribution to spinach chloroplast dark metabolism. Methods for estimating the OPP contribution have been described (23). Method I uses the relation between the total $[^{14}C]CO_2$ release and the total ^{14}C accumulation

as triose-P and PGA. The summed CO_2 release and anion accumulation can be taken as representing the total activity of glycolysis and the OPP cycle, since significant accumulation is only seen in triose-P and PGA among the anions (Fig. 4). One triose-P is formed for every three CO_2 released in the OPP cycle; hence, the OPP flux can be estimated as twice the CO_2 release. The OPP cycle contribution is then given by double the CO_2 release divided by the sum of the CO_2 release and anion accumulation. Method II uses the relation between [^{14}C]CO_2 release from [1-^{14}C]glucose and [2-^{14}C]glucose. Katz and Wood (7) have derived a relation between the OPP contribution and the specific radioactivity of the C-1 of the HMP in samples suplied with [1-^{14}C]glucose and with [2-^{14}C]glucose. By determining these specific activities, the OPP cycle contribution can be found. In chloroplasts, all the CO_2 is released *via* the OPP pathway. Thus, the relative release of [^{14}C]CO_2 from [1-^{14}C]glucose and [2-^{14}C]glucose gives a direct estimate of the relative specific radioactivity of the C-1 of the HMP in the chloroplasts, and, hence, can be used to estimate the OPP contribution.

These methods can be applied to the data obtained in the presence of 5 mM phosphate. An estimate of 17% OPP activity is given by method II and Table V, and 23% is given by method I and Figure 5. The correct answer lies between these estimates, as release of [^{14}C]CO_2 by net recycling of triose-P will give an overestimate by method I and an underestimate by method II. The presence of L-pathway of the OPP cycle would also lead to an underestimate by method II but would not affect method I. An activity of between 17 and 23% is similar to that found in pea choroplasts (23) and means that, of the glucose 6-P metabolized, 37 to 47% will be converted to 6-P gluconate by glucose 6-P dehydrogenase, and the rest will be transformed by phosphofructokinase (23). These activities are comparable with those found in mammary tissue and adipose tissue (5, 6) when a high activity of the OPP cycle is required to supply NADPH for fat synthesis and is considerably higher than that found so far in other plant tissues (1).

Response of Starch Mobilization to Pi. Table VI shows the influence of Pi on starch mobilization. When Pi is omitted, accumulation of anions (representing phosphorylated compounds) is prevented, and 80% of the starch degradation leads to free sugars. Apparently, there is a switch from phosphorolysis to hydrolysis. As the decreased production of anions is partially balanced by increased formation of neutral sugars, it seems that omitting Pi does not greatly lower the overall rate of starch breakdown in these chloroplasts.

The increased phosphorolysis produced by adding Pi is accompanied by a rapid formation of triose-P and 3PGA (see above), accounting for up to one-half of the products of starch. This is due to stimulation of glycolysis rather than stimulation of the OPP cycle. The lowered CO_2 release during starch mobilization (Table VI) and during metabolism of [U-^{14}C]glucose (Table III) reveals that the activity of the OPP pathway is even decreased when Pi is added. Furthermore, by using method II (23), it can be estimated from the decreased C-1:C-2 ratio in CO_2 (Table V), that the percentage of the HMP metabolized in the orthodox OPP cycle rises from about 17 to 26% when Pi is omitted. In addition, there is a very sharp rise in the release of [^{14}C]CO_2 from [6-^{14}C]glucose relative to that from [1-^{14}C]glucose or [2-^{14}C]glucose, indicating that, additionally, either the L-path or triose-P cycling are also markedly stimulated. This may be because a restriction of triose-P export, due to lack of external Pi (2), leads to accumulation of triose-P in the stroma favoring the aldolase reactions producing fructose 1,6-bisP or octulose 1,8-bisP, key intermediates for triose-P cycling and for the L-path, respectively.

A restriction of phosphorolysis, but not of hydrolysis, in the absence of Pi has previously been described in spinach chloroplasts (3, 15), but the full effect of Pi on all the various pathways was not so clearly shown, because CO_2 formation could not be measured. In these earlier experiments, the overall starch breakdown was more strongly inhibited when Pi was omitted, and no stimulation of hydrolytic breakdown was seen. This might, perhaps, reflect a difference between chloroplasts with high and those with low starch contents.

The response of chloroplast starch mobilization to changing Pi concentrations in the medium occurs gradually over a range varying from 50 μM to 1 mM (Stitt, unpublished). *In vivo*, the precise effects will be modified by the presence of PGA and triose-P, as well as Pi, in the cytosol. It is reasonable to suggest that, as extrachloroplastic demand for respiratory substrates increases, the triose-P and PGA levels in the cytosol will fall and the Pi will rise. This should increase the net flux of Pi into the chloroplasts and that of triose-P and PGA out of the chloroplast and, hence, increase the net flux from starch *via* glycolysis through to exported phosphorylated products. When there is no cytosolic demand for phosphorylated intermediates and cytosolic Pi declines, phosphorolytic starch mobilization would continue at a much lower rate, providing mainly substrates for chloroplast energy metabolism, with much of the HMP now being metabolized in the OPP cycle. More of the starch will now be degraded hydrolytically and exported as free sugars to the cytosol.

Table VI. *The Dependence of the Metabolism of Products of Starch Metabolism in Starch-Loaded Spinach Protoplasts on the Presence of Phosphate in the Medium*

The experiments with 5 mM Pi are taken from Table II. The data for nil Pi are taken from parallel incubations carried out with the same protoplast preparations. The results are mean values of seven experiments.

Pi	Starch breakdown	Accumulation of		
		Neutrals	Anions	CO_2
mM		*μatom C/mg Chl·h*		
5	10.4	5.0	4.6	0.8
0	9.0	6.3	0.1	1.6

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