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SIMULTANEOUS SYNTHESIS AND DEGRADATION OF STARCH IN SPINACH CHLOROPLASTS IN THE LIGHT

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The rates of starch breakdown in isolated starch-loaded spinach chloroplasts have been measured in the dark and light at a range of phosphate concentrations, and compared with the rates of starch synthesis occurring simultaneously in the light. (1) Illumination has little inhibitory effect on starch breakdown when chloroplasts are suspended in medium containing a high phosphate level. At low phosphate levels, up to 67% inhibition is observed. (2) CO_2 evolution is prevented by illumination at all phosphate levels, showing that the oxidative pentose phosphate pathway has been inhibited. (3) The rates of starch degradation in the light are significant compared with the rates of starch synthesis, even at phosphate levels which are optimal for photosynthesis or for starch synthesis. (4) It is concluded that net starch accumulation in the light represents a balance between synthesis and breakdown. This balance is very sensitive to the rate at which carbon is withdrawn from the chloroplast in exchange for P_i.

Introduction

Photosynthesis in chloroplasts involves conversion of CO_2, H_2O and P_i into triose phosphate [1]. Most of the triose phosphate is exported via the phosphate translocator in exchange for P_i [2] and is used to synthesize sucrose in the cytosol [3–5]. During sucrose synthesis the P_i is released and can be cycled back into the chloroplasts. Some fixed carbon can also be temporarily retained in the chloroplasts as starch. In isolated chloroplasts starch accumulation is enhanced when export of triose phosphate in exchange for P_i is restricted [6,7]. Probably, in vivo, when carbon fixation exceeds the rate at which triose phosphate can be converted into sucrose in the cytosol, the chloroplasts convert an increasing proportion of the triose phosphate into starch [8]. In this way the esterified phosphate group is released within the stroma, and is then directly available for further photosynthesis. Thus, starch might be seen as a buffer to sucrose metabolism. A plant grows under rapidly changing environmental conditions and the rate of photosynthesis varies correspondingly. It would be wasteful for a plant to possess a capacity for the synthesis and export of sucrose which matches the highest rates of photosynthesis it attains. Instead, some carbon can be temporarily retained in the leaf as starch to be degraded later and, at least in part, converted to sucrose [9,10]. This extends the synthesis and export of sucrose over a longer period and may economize, for example [11,12] in the quantity of phloem which must be formed and maintained.

The daily rise and fall of starch levels [9,10,13–16] in the leaf must involve rapid changes in the relative activities of the synthetic and degradative pathways [17]. It can be asked whether synthesis and degradation are temporally separated and occur only in the day and night, respectively, or whether their

Abbreviations: Chl, chlorophyll; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

interaction with photosynthesis is more complex. It has long been known that starch can be synthesized from high concentrations of sugar in the dark (see Ref. 18). It is not known whether significant starch degradation can occur in the light, either as an alternative to synthesis, or even simultaneously. Recently, we have described how spinach leaf protoplasts can be used to isolate intact, starch-loaded chloroplasts which degrade their starch at physiological rates of about 10 μatom C/mg Chl per h [19]. We have now adapted this method to compare the rates of starch synthesis in the light with the rates of starch degradation in the light and the dark.

Methods

Spinach (*Spinacia oleracea* L., U.S. Hybrid 424, Ferry Morse Seed Co., Mountain View, CA) was grow in water culture under a 10 h light/14 h dark cycle [20]. Illumination was summer light, supplemented with a mixture of fluorescent (HqI, 250 W, Osram) and tungsten lamps (Concentra, 150 W, Osram) yielding an additional illumination of 15 W/m^2. The temperature was 24–27°C in the light and 15–20°C in the dark.

Protoplasts were prepared as previously described [19] from leaves of plants which had been preilluminated for 8–10 h. The labelling of starch during photosynthetic $^{14}CO_2$ fixation by protoplasts, isolation of chloroplasts from these protoplasts, analysis of the distribution of radioactivity, and measurement of substrates by enzymic analysis have been described [19]. One modification was included for experiments on the effect of light (see Scheme I). After releasing chloroplasts from protoplasts, the chloroplasts were sedimented by centrifugation (5 min, 200 × *g*), resuspended in 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, 1 mM 3-phosphoglycerate, 5 mM $NaHCO_3$, and illuminated (180 W/m^2) for 10 min before being applied to a discontinuous Percoll density gradient.

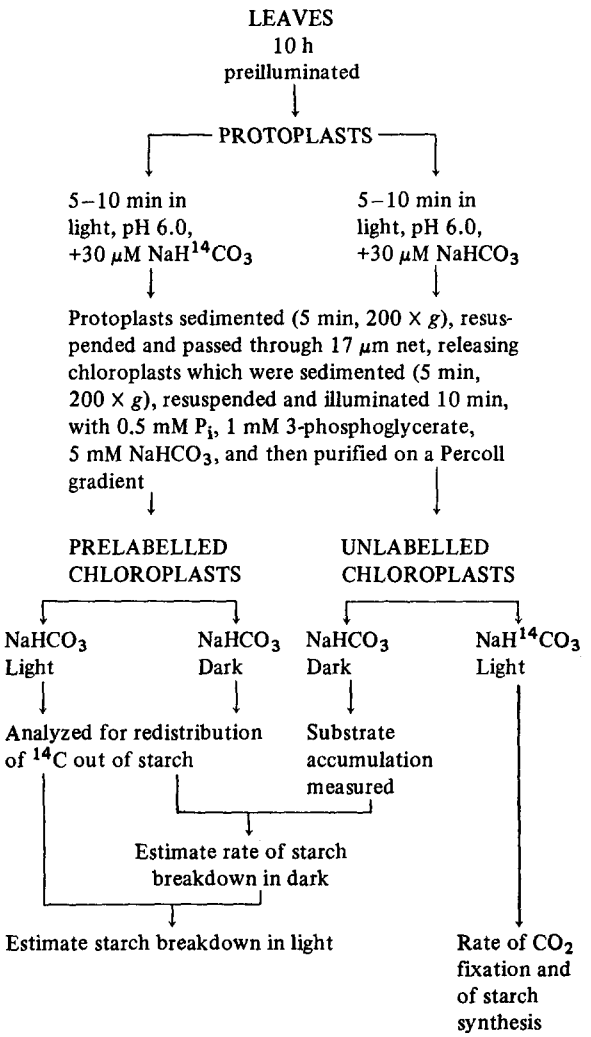
For experiments investigating the effect of light and dark on starch degradation and synthesis all incubations were carried out at 5–10 μg Chl/ml in 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 12 mM $NaHCO_3$. Illumination was with red light (G 630 cut-off filter, Schott, Mainz, F.R.G., 100 W/m^2). P_i and $H^{14}CO_3^-$ were included as specified in Results and Discussion. Conditions in experiments investigating the effects of pH and dithiothreitol are specified in Fig. 2 and Table II, respectively.

Results and Discussion

The measurement of starch breakdown and synthesis simultaneously

Normally, the measurement of simultaneous fluxes in two directions is complicated and involves the use of dual-labelled isotopes to study turnover within intermediate pools [21]. In isolated chloroplasts, the problem is simplified because the products of starch breakdown accumulate outside the chloroplasts as end products of which the formation can be measured, and the synthesis of starch can also be measured as accumulation of an end product. To measure the synthesis of starch, $^{14}CO_2$ of known specific radioactivity is supplied to nonradioactive chloroplasts, and the accumulation of ^{14}C in starch is monitored. Starch breakdown can be measured in the dark by monitoring the flow of ^{14}C out of starch into identified end products and then using enzymic analysis to provide an absolute rate of accumulation in these products [19]. This procedure is complicated in the light because almost all the products of starch breakdown are simultaneously formed at a much faster rate in the Calvin cycle, so that their rate of formation from starch cannot be estimated directly by enzymic analysis. Therefore, in the light, only the movement of ^{14}C out of prelabelled starch into products can be measured. This flux of radioactivity is then calibrated by comparing it with the release of ^{14}C and the absolute rates of product formation from starch breakdown in the dark. This approach involves the reasonable assumption that the specific radioactivity of the starch degraded in the light and the dark is similar.

Protoplasts were prepared from preilluminated leaves so that they contained high levels of starch [19]. They were divided into two samples (Scheme I) and allowed to photosynthesize with 30 μM bicarbonate at pH 6 for about 10 min until the bicarbon- was fully incorporated. In one sample, $H^{14}CO_3^-$ was included, so that the starch was prelabelled. The prelabelled chloroplasts were then isolated from the protoplasts. During this procedure all the exchange-



Scheme I. Flow diagram of experimental protocol to investigate simultaneous rates of synthesis and degradation of starch, in light and dark. For details see Methods, Table I, Fig. 1 and Ref. 19.

able, ^{14}C-labelled intermediates were removed from the chloroplasts by incubating them for 10 min in the light with 1 mM 3-phosphoglycerate. These ^{14}C-labelled intermediates could otherwise have moved back into starch in the light, masking any movement of ^{14}C out of starch. This treatment did not alter the pattern of ^{14}C-labelled starch mobilization. After isolation of the ^{14}C-labelled chloroplasts, one sample was quenched immediately, and one after 30 min in the light or dark. Chloroplasts were prepared from the unlabelled protoplasts in exactly the same way. Some of these chloroplasts were incubated in the light with $H^{14}CO_3^-$ in order to measure starch synthesis and CO_2 fixation. The remainder of the chloroplasts were incubated in the dark, without radioactivity, and then quenched, before determining the accumulation of phosphorylated intermediates and glucose formed during starch breakdown using enzymic analysis. These experiments involved comparing measurements in separate incubations in labelled and unlabelled chloroplast preparations. It is stressed that the conditions throughout their preparation and incubation were identical except for the presence or absence of $H^{14}CO_3^-$.

The details of an experiment on starch breakdown in the light and in the dark are presented in Table I. The ^{14}C-labelled degradation products from labelled starch were separated into precipitable starch, volatile CO_2 and anionic and neutral compounds, as obtained by a combination of anion and cation exchangers [5]. The degradation products from unlabelled starch were assayed by enzymic analysis. These include hexose monophosphates, fructose 1,6-bisphosphate, triose phosphate and 3-phosphoglycerate, which are summed as anions, and also glucose and maltose. It has been shown earlier that the labelled and unlabelled products of starch breakdown in the dark show a similar distribution, indicating that these products are equally labelled [19]. It is therefore possible to employ enzymic analysis for a quantitative determination of starch degradation from ^{14}C measurements. As has been discussed above, in the light the starch degradation products cannot be assayed enzymically. The estimation of the rates of starch degradation product formation in the light from ^{14}C radioactivity measurements is therefore based on the assumption that the starch degradation products in the light are also labelled equally, and with the same specific radioactivity as those released in the dark. In the light this assumption is difficult to check but two arguments suggest that it is valid. First, the similarity between the ^{14}C distribution in the light and dark, except for CO_2 (see later), suggests that the overall pattern of starch mobilization remains similar. Second, it was possible to measure glucose formation by enzymic analysis in the light. When high P_i or DL-glyceraldehyde levels were added in order to restrict photosynthesis, similar levels of glucose were found

TABLE I

ESTIMATION OF STARCH BREAKDOWN IN LIGHT AND DARK

Labelled and unlabelled chloroplasts were prepared from protoplasts (see Methods and Scheme I) and aliquots from both were immediately quenched with 10% $HClO_4$. The remainder of the unlabelled chloroplasts were incubated in the dark, and the rest of the labelled chloroplasts were divided and incubated either in the light or dark. After 30 min the samples were quenched with 10% $HClO_4$. All incubations included 5 mM P_i. From the labelled extracts, the radioactivity in starch, CO_2, neutral and anionic compounds was measured [19]. In the unlabelled extracts, substrates were measured by enzymic analysis [19]. Hexose monophosphates, fructose 1,6-bisphosphate, triose phosphate and 3-phosphoglycerate are summed as anions.

	dpm/incubation			Change in ^{14}C, as % of the change in starch in the dark, during 30 min		Accumulation of products in dark measured by substrate analysis (μatom C/mg Chl per h)	Estimated formation of starch degradation products (μatom C/mg Chl per h)	
	0 min	30 min						
		Dark	Light	Dark	Light		Dark	Light
Starch	8 508	3 642	4 475	100	83	–	17.1	14.2
CO_2	420	1 029	435	13	0.3	–	1.9	0.05
Anionic compounds	1 204	3 197	2 801	41	33	7.7	–	6.3
Neutral compounds	1 204	3 114	3 005	39	37	–	7.3	7.0
Glucose	–	–	–	–	–	5.3	–	–

to accumulate in the light and dark (not shown). With lower P_i levels added to the medium only about two-thirds as much glucose accumulated in the light. Similar relationships are also found in the flow of ^{14}C into neutral products under these conditions (Table I and below) suggesting that ^{14}C acts as a valid marker for flow of carbon.

When the P_i level in the medium is high, this approach gives a reliable estimate of the rate of starch breakdown, since the ^{14}C-labelled intermediates released from starch are rapidly exported from the chloroplasts and diluted into the surrounding medium. In medium containing a low P_i level, however, such export is slower and some recycling of ^{14}C back into starch in the light may occur. For this reason, the rates estimated in the light, when export of phosphorylated intermediates is restricted, should be considered as minimal estimates of the rate of starch breakdown.

To measure starch synthesis, samples were taken at 0, 10, 20 and 30 min to ensure that photosynthesis and starch synthesis continued throughout the entire incubation. In order to allow continued photosynthesis at low P_i concentrations in the medium, the experiments were all carried out at low chlorophyll concentrations of 5–10 μg Chl/ml. A possible source of error in these measurements arises if starch degradation continued in the light. In this case ^{14}C may be incorporated into starch and remobilized immediately afterwards, thus leading to an underestimate of the rate of synthesis. However, the effects of such recycling are not likely to alter qualitatively any conclusions drawn from this method. Considerable unlabelled starch is degraded along with labelled starch, since comparison of ^{14}C movement and carbon flow shows considerable isotope dilution (Stitt, unpublished data). In any event, the rate of degradation would have to be high compared to that of synthesis before such recycling could greatly alter the rate of ^{14}C accumulation in starch.

The influence of illumination and phosphate levels on starch breakdown

Because P_i is implicated in the control of starch synthesis and degradation [6–8,17,19], the effect of light on starch degradation was investigated at a range of P_i concentrations in the medium. As previously shown [19], starch mobilization in the dark is itself influenced by the P_i level. With suboptimal P_i concentrations in the medium, on average a 13% inhibition of total starch degradation is observed [19]. Under these conditions, the formation of phosphorylated

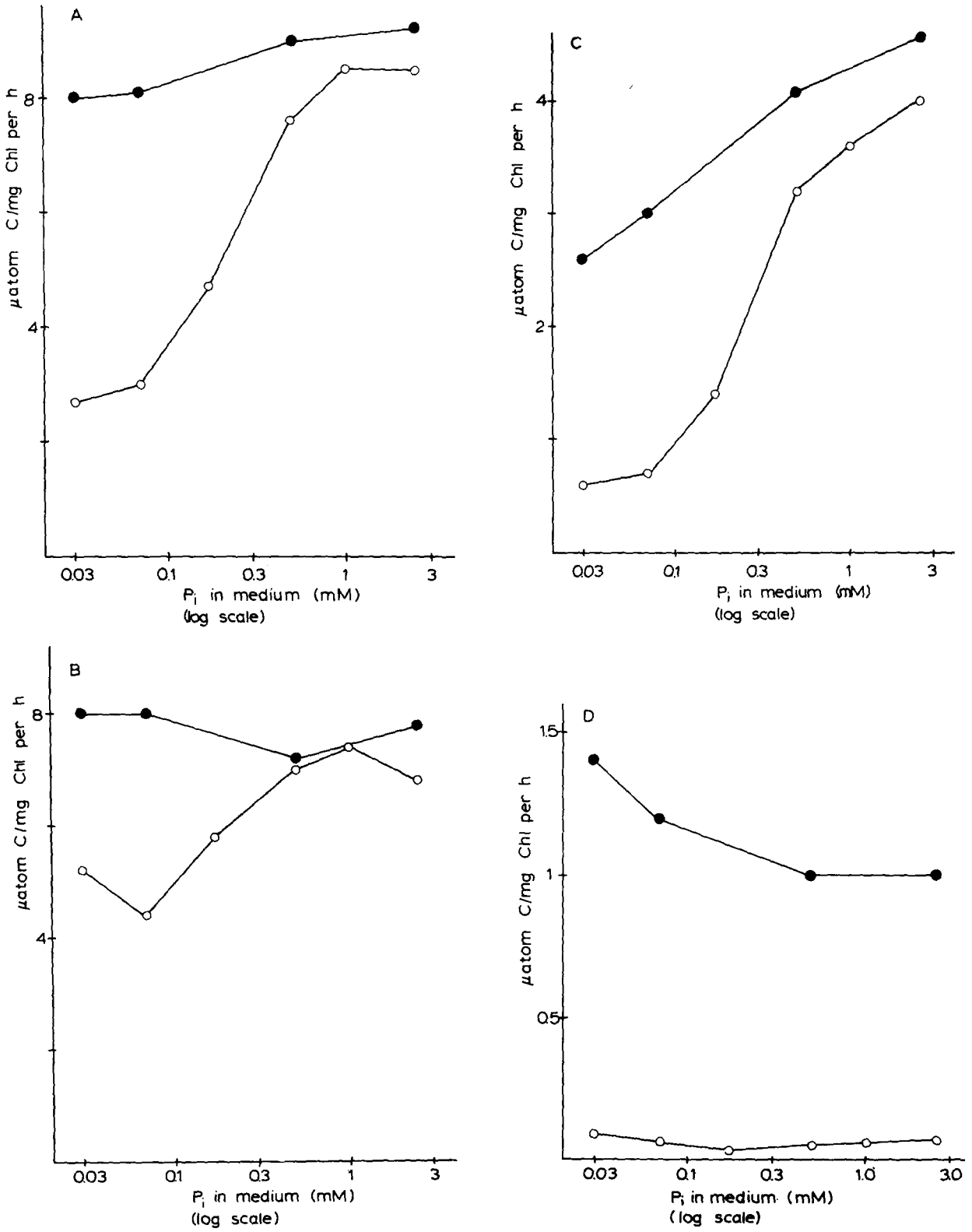


Fig. 1. The effect of illumination on starch breakdown. Labelled and unlabelled chloroplasts were prepared as for Table I and quenched with $HClO_4$ immediately, or after 30 min in darkness (unlabelled) or light or darkness (labelled). The P_i level was varied as shown. Unlabelled extracts were assayed for glucose and for hexose monophosphate, fructose 1,6-bisphosphate, triose phosphate and 3-phosphoglycerate (anions). Labelled extracts were separated into starch, CO_2, neutral compounds and anions. The rates of breakdown were extrapolated for the various products in light and dark. (A) Total starch breakdown, (B) accumulation of neutral compounds, (C) accumulation of anions, (D) release of CO_2. The results are the mean of three separate experiments with different protoplast preparations; in each experiment every treatment was carried out in triplicate. (●——●) Dark, (○——○) light.

starch degradation products is markedly lowered, whereas the release of CO_2 and of sugars (such as glucose) can even be slightly enhanced [19]. Apparently, a lowering of the P_i level results in a switch from phosphorolytic to hydrolytic starch breakdown.

The influence of light on starch breakdown is shown in Fig. 1, where the results of three separate experiments with different protoplast preparations are averaged. The effect of light depends on the P_i concentration. With high levels of P_i, light has only a small inhibitory effect on the total starch breakdown, mainly due to a slightly decreased formation of phosphorolytic (anionic) starch degradation products, as the formation of neutral compounds is only slightly affected. This is different with low P_i levels, where starch breakdown is markedly inhibited by light. In this case, not only the formation of phosphorylated (anionic) compounds but also that of the products of hydrolytic starch degradation (neutral compounds) are markedly depressed. The latter inhibition is unlikely to be an artefact due to cycling of ^{14}C because in spinach the rates of starch synthesis out of glucose are very low [7]. The marked inhibition of phosphorolysis during active photosynthesis is probably due to the accumulation of intermediates and the lowering of the stromal P_i concentration which accompanies the onset of carbon fixation in isolated chloroplasts [22].

Effect of pH and thiol agents on starch degradation

Since light had little effect on the rate of starch mobilization in the presence of high P_i levels, it seemed likely that the pH and the reductive state of electron-transport carriers, e.g., thioredoxin, on their own, do not play a major role in controlling starch degradation. This was verified by investigating the effect of these parameters on starch degradation in the dark. Dithiothreitol, a commonly used substitute for reduced thioredoxin [23], when added to chloroplasts in the presence of 5 mM P_i did not inhibit starch breakdown (Table II). It did, however, strongly inhibit dark CO_2 release, demonstrating that it had actually entered the chloroplast. Chloroplast starch degradation shows a gradual response to the pH of the medium in the presence of high P_i levels (Fig. 2). An optimal rate is found at pH 7.3, but at pH 8.1, 60% of this rate is still observed. The alkalinization of

TABLE II

EFFECT OF DITHIOTHREITOL ON CHLOROPLAST STARCH MOBILIZATION IN THE DARK

Labelled chloroplasts were prepared from protoplasts (see Methods) and some were quenched immediately with 10% $HClO_4$. The remainder were incubated in the dark (30 μg Chl/ml) in 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM P_i and dithiothreitol as specified, and were quenched after 30 min. Radioactivity in starch, CO_2, anionic and neutral compounds were measured (Methods), and the change during the 30 min incubation calculated. Each treatment was triplicated.

Dithiothreitol	Change in radioactivity (dpm/incubation) (×10^2)			
	Decrease in starch	Increase in		
		Anions	CO_2	Neutral compounds
0	144	75	13	56
5	144	66	2	76

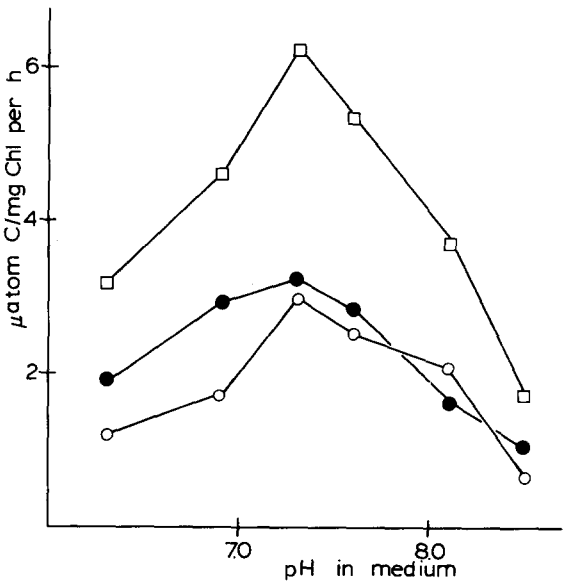


Fig. 2. The influence of pH on starch mobilization in the dark. Unlabelled chloroplasts were prepared from protoplasts. 10% $HClO_4$ was added immediately or after 30 min in the dark in 0.33 M sorbitol, 50 mM Hepes, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 5 mM KH_2PO_4 at the pH shown. The samples were neutralized and enzymatically assayed for substrate accumulation (see Methods). (□——□) Total, (●——●) phosphate esters, (○——○) glucose.

the stroma occurring after illumination of chloroplasts [24] could therefore only restrict, but not totally inhibit, starch degradation in the light. These observations are in agreement with the pH dependence observed for the isolated chloroplast endoamylase [14,25] and phosphorylase [26]. Furthermore, no evidence for modulation by thioredoxin or dithiothreitol could be found for either isolated enzyme [25,26].

The effect of light on the oxidative pentose phosphate pathway

It has previously been shown that CO_2 is released during starch mobilization in isolated chloroplasts, via the oxidative pentose phosphate pathway [19,27]. As shown in Fig. 1D, the dark release of CO_2 is maximal at low P_i levels (see also Ref. 19). Apparently, the activity of the oxidative pentose phosphate pathway rises when a lack of exchangeable P_i in the external medium restricts export of carbon from the chloroplasts.

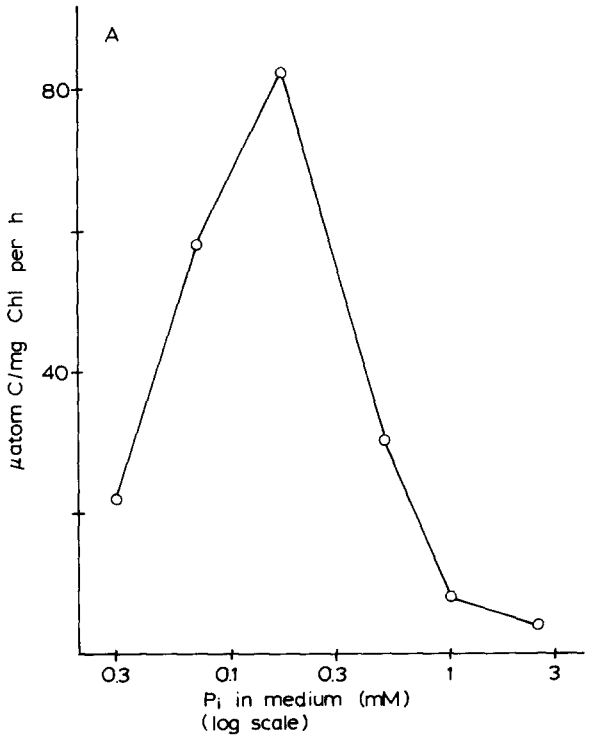
Illumination almost totally inhibits CO_2 release at all P_i concentrations (Fig. 1D). It is improbable that this is an artefact due to refixation of the released CO_2, since the chloroplasts were incubated with 12 mM bicarbonate and of this only a minute fraction was fixed. Such a refixation would be dependent on P_i concentration and, as generally observed for CO_2 fixation, it should have a marked optimum at 0.17 mM P_i (see Fig. 3). In contrast, CO_2 release in the light is found to be very low over the entire range of P_i concentrations employed (Fig. 1D). The activity of the oxidative pentose phosphate pathway remaining in the light is negligible compared to photosynthetic fluxes. One CO_2 molecule is eventually released for every glucose 6-phosphate molecule oxidized by glucose-6-phosphate dehydrogenase. The activity of this enzyme in the light is therefore less than 0.1 μmol substrate/mg Chl per h. In comparison, during photosynthesis at about 100 μmol CO_2/mg Chl per h, the activities of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase must be 30–40 μmol/mg Chl per h.

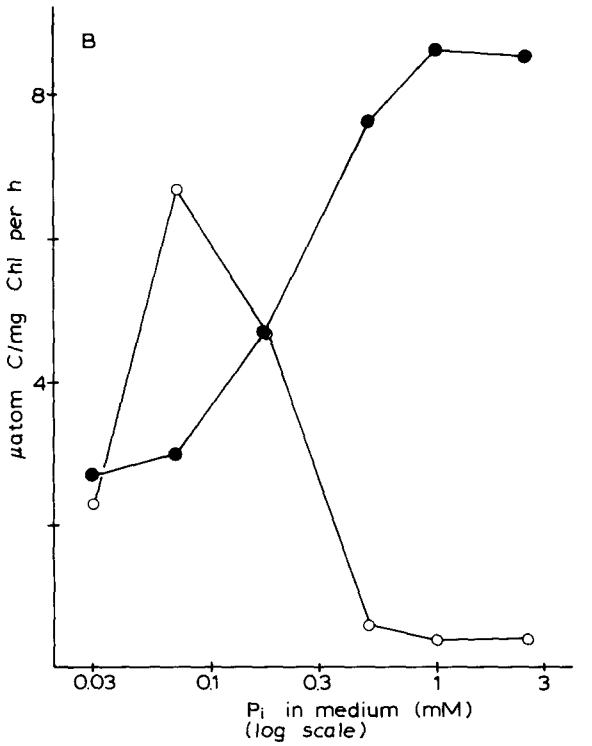
The question arises as to how glucose-6-phosphate dehydrogenase is inactivated in the stroma. This enzyme is known to be deactivated in the light, where the deactivation can be simulated by the addition of dithiols, such as thioredoxin or dithiothreitol [23,28, 29]. Thus, also in our experiments, CO_2 evolution in the dark is inhibited 85% by the addition of dithiothreitol (Table II). Apart from this light inhibition, the enzyme is also sensitive to inhibition by increased NADPH/NADP ratios. Although the increased stromal pH in the light increases the activity of the pure enzyme [30] and of the enzyme in a reconstituted stromal system [31,32], the change in the NADPH/NADP ratio between dark and light is probably enough, on its own, to inhibit the enzyme [30]. Possibly, both direct light inactivation and the NADPH/NADP ratio are capable of switching the enzyme off in the light. The NADPH/NADP ratio will also be important in controlling the oxidative pentose phosphate cycle activity in the dark. It can be shown that adding phenazine methosulphate to chloroplasts in the dark in order to oxidise NADPH markedly stimulates the oxidative pentose phosphate cycle (Stitt, unpublished data).

Rates of synthesis and degradation of starch in the light

The average rates of CO_2 fixation, starch synthesis and starch degradation in the light are shown in Fig. 3. The data are from the same three experiments which are shown in Fig. 1. As previously shown [6,7], the optimum P_i level for starch synthesis (0.07 mM) is lower than that for CO_2 fixation (0.17 mM). This can be explained by a stimulation of ADP-glucose pyrophosphorylase [33] by the increased 3-phosphoglycerate/P_i ratio in the stroma when the external P_i level is low [7].

In these starch-loaded chloroplasts, the efflux of carbon out of starch is high compared to that into starch, even under those conditions (i.e., low P_i concentration) favouring maximal photosynthesis or starch synthesis. Only at 0.07 mM P_i is the rate of starch synthesis higher than the estimated rate of breakdown in the light. This P_i concentration is the optimum for starch synthesis. At 0.17 mM P_i, which supported the highest rate of photosynthesis, the rates of starch synthesis and degradation are similar. At other P_i levels degradation exceeds the rate of synthesis. Since increased P_i concentration simultaneously inhibits starch synthesis and stimulates starch degradation, the net accumulation of starch will be even more sensitive to the level of P_i than was indicated by studies of the synthetic pathway alone. By altering





the rate at which carbon leaves the chloroplast in exchange for P_i, large changes can be produced in the balance of synthetic and degradative reactions, and this balance in turn determines the rate of net starch accumulation.

Of course, energy is lost in a futile cycle when starch is being simultaneously synthesized and degraded, but in terms of the total energy being used during photosynthesis, it is not a great added expense. In the experiments shown in Fig. 3, about 6% of the total photosynthate is converted into starch (chloroplasts in 0.07 mM P_i). Each carbon atom fixed requires three molecules of ATP to be expended in the Calvin cycle. A hexose phosphate molecule can be incorporated into starch, released by hydrolysis and rephosphorylated at the cost of three molecules of ATP, or 0.5 molecules ATP/atom C. If 6% of the total carbon fixed is entering starch and half of this is recycled, the loss of ATP would be $(3 \times 0.5/100 \times 3) \times 100$ or 0.5% of the ATP used in the Calvin cycle.

On the metabolism of starch in leaves

Caution is needed when extrapolating from isolated chloroplasts to leaves, especially when interactions between the chloroplast and its environment are involved, as in these experiments. However, there is some independent evidence that there can be an efflux of carbon out of starch in leaves in the light. When tobacco [34] or cottonwood [35] leaves were pulsed with $^{14}CO_2$ and then given a light chase, radioactivity decreased in the starch although the synthesis of starch continued. When pea leaves are illuminated continuously, they accumulate starch up to about 20 h, but the total level of starch then falls, showing that degradation is now even exceeding syn-

Fig. 3. Comparison of photosynthesis, starch synthesis and starch breakdown in the light at varying P_i levels. Chloroplasts were prepared from protoplasts and illuminated in 0.33 M sorbitol, 50 mM Hepes (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, catalase (500 U/ml), 12 mM $NaHCO_3$ and P_i as shown at 5–10 μg Chl/ml. Samples were withdrawn from incubation containing $^{14}HCO_3^-$ (6 Ci/mol) into $HClO_4$ at 0, 10, 20 and 30 min to measure CO_2 fixation and starch synthesis. The experiment was carried out with the same three protoplast preparations as used for Fig. 1. (A) Rate of CO_2 fixation at maximum. (B) Rate of starch synthesis (○———○) at maximum. The rate of starch degradation (●———●) over 30 min is taken from Fig. 1, for comparison.

thesis in the light [36]. Similar results have been found for spinach [14]. When barley, oats or rye were grown in sunlight during the summer in Munich, the levels of starch in the leaves began to fall at about 3 p.m. (Stitt and Birnbacher, unpublished data). Although such results do not reveal the exact fluxes in and out of starch they do indicate that such fluxes can occur simultaneously, and also that degradation can exceed synthesis in leaves under at least some conditions in the light. Thus, the extent of starch accumulation in leaves may depend not only on the rate of starch synthesis, but also on the interaction of the fluxes into and out of starch.

In leaves this balance of synthesis and degradation of starch may be regulated by a number of factors. In the long term, the partitioning of photosynthate between starch and sucrose would depend upon the overall enzyme, transport and storage capacities of the leaf. This partitioning appears to be controlled. For example, when plants were grown at a lower light intensity or under a shorter light period, more of their photosynthate was diverted into starch [11,12]. If starch were just an overflow product accumulating when sucrose synthesis cannot accommodate all of the photosynthate, then these treatments should have decreased, not increased, the relative importance of starch. It was argued [11,12] that a certain amount of starch accumulation is of advantage and that the amount of starch accumulated can be selected, although the mechanisms involved are as yet unknown.

In the short term there are also rapid changes in the metabolism of starch, including both the usual alternation between accumulation in the day and depletion in the night, and alterations in the metabolism of starch produced by factors including light intensity, CO_2, temperature and the levels of phosphate and sucrose in the leaf [7,8,15,37,38]. Flexible use of starch as an alternative to sucrose under varying conditions would need fine control, operating within the limits set by the overall capacities of the leaf. Several factors are implicated in such fine control.

Modification of enzymes would provide one way of switching between accumulation and depletion of starch. However, thioredoxin, or similar systems, do not appear to be involved in regulating the enzymes of starch degradation (see above), in contrast to the oxidative pentose phosphate pathway. Oscillations in the extractable activity of the chloroplast amylolytic enzymes do occur in leaves [14] but these oscillations are slow, occurring over hours, and produce only a 2-fold change in activity. The cause of these oscillations is unclear, since they continue even in continuous light or darkness. Their precise role in controlling starch mobilization is also unclear. After turning the light off, the maximal rates of starch mobilization are reached before there is a significant increase in the extractable amylase activity, showing that the activity present in the light is more than adequate to support the highest rates of starch mobilization.

Another means of regulation is provided by changes in the stromal levels of ions and substrates, which can modify the catalytic activity of enzymes. The balance between P_i and phosphorylated intermediates is of particular importance. An increase in the 3-phosphoglycerate/P_i ratio enhances starch synthesis [7] by stimulating ADP-glucose pyrophosphorylase [33]. Simultaneously, starch breakdown would be restricted by the lowered P_i level [6,7], especially in the light (Figs. 1 and 3). The stromal P_i and 3-phosphoglycerate levels probably depend upon how much P_i is available in the cytosol for import into the chloroplast in exchange for triose phosphate. In turn, the levels of P_i and phosphorylated intermediates in the cytosol may reflect the extent to which triose phosphate is being converted to sucrose [8]. This would provide a gradual and fine coupling between starch and sucrose metabolism, although studies of the properties of the cytosolic enzymes and of subcellular metabolite levels are needed to establish the precise mechanism by which this coupling takes place.

In transitions between the light and dark, however, the 3-phosphoglycerate/P_i ratio does not seem to play an important role. In isolated chloroplasts, the ratio changes too slowly to account for the cessation of starch synthesis in the dark [39]. When barley protoplasts are darkened, the ratio even rises, while starch synthesis stops (Stitt, unpublished data). On the other hand, the alkalinization of the stroma and the increased ATP/ADP ratio in the light may be involved in the control of starch metabolism. Starch synthesis is decreased at a low ATP/ADP ratio [40], which inhibits ADP-glucose pyrophosphorylase [33]. How-

ever, the ATP/ADP ratio does not always change markedly between light and dark (see Ref. 5) and more measurements are needed in order to understand the contribution of these factors in leaves. Also, since starch can accumulate in the dark when exogenous sugars are supplied [8], the inhibition of synthesis in the dark is reversible. How this occurs is unknown.

The amount of starch may itself also influence the rates of starch synthesis and breakdown. Starch accumulation often slows down or even stops towards the end of the light period [8,10,13]. The experiments presented in Figs. 1 and 3 were carried out with starch-loaded chloroplasts. Lower rates of starch degradation might be expected in chloroplasts isolated at the start of the day with less starch, and this would lead to a change in the balance between synthesis and degradation.

Although starch normally accumulates in the light and is depleted in the dark, synthesis and degradation can occur simultaneously. In the light, the balance between them is sensitive to the rate at which carbon is withdrawn from the chloroplast in exchange for P_i. If starch is a temporary storage product which acts as a buffer to sucrose, it is to be expected that both the P_i balance is important in determining whether starch is accumulated or mobilized, and also, that starch synthesis and degradation are not temporally segregated between the day and night by the direct effects of illumination on the availability of reducing groups, stromal pH, Mg^{2+} concentration and adenylate ratios. This gradual control of starch metabolism can be contrasted with the rapid and almost complete inactivation of the Calvin cycle in the dark due to the absence of reducing groups, and the decreased pH, Mg^{2+} concentration and adenylate ratios in the stroma [41]. This strict control of the Calvin cycle by a light-dependent on-off switch is essential if starch breakdown in the dark is to provide substrates for respiration and sucrose synthesis.

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