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Subcellular Metabolite Levels in Spinach Leaves[1]

REGULATION OF SUCROSE SYNTHESIS DURING DIURNAL ALTERATIONS IN PHOTOSYNTHETIC PARTITIONING

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ABSTRACT

The alterations of subcellular metabolite levels during the day in spinach leaves have been investigated using nonaqueous density gradient centrifugation to separate chloroplasts, cytosol, and vacuole. The results provide direct evidence for the role of sucrose phosphate synthase and cytosolic fructose 1,6-bisphosphatase in regulating sucrose synthesis in leaves and also show that the phosphate translocator is kinetically limiting *in vivo*.

Recently, progress has been made in understanding how photosynthetic sucrose synthesis is regulated in spinach leaves in response to the supply of photosynthate and the demand for sucrose (Fig. 1). As photosynthesis increases, trioseP[2] becomes available for synthesis of sucrose in the cytosol, and there is a decrease of $Fru2,6P_2$ (32), a regulator metabolite which is a potent inhibitor of the cytosolic Fru1,6Pase (16). The increased substrate and decreased inhibitor concentration activates the cytosolic Fru1,6Pase. The increased production of hexoseP is, in turn, thought to stimulate SPS, via a rising GLc6P/Pi ratio (6, 18, 19). In addition, there is a light-activation of SPS in spinach leaves (40). On the other hand, when sucrose accumulates in the leaf the rate of sucrose synthesis can be decreased, and more photosynthate is diverted to starch in the chloroplast (3, 17, 18, 34, 38, 40). A 20 to 40% increase of the overall content of hexoseP, UDPglucose, and trioseP in spinach leaves (30, 33) shows both SPS and the cytosolic Fru1,6Pase are being inhibited *in vivo*. This is caused by a decrease in the amount of SPS activity (40) and a 2- to 3-fold increase of $Fru2,6P_2$ (17, 30, 33). Evidence is accumulating for a similar regulation in other plants. $Fru2,6P_2$ decreases after illumination in pea (2), maize (34), and barley (M Stitt unpublished data) leaves. Distinct and rapid alterations of SPS activity are found during light-dark transitions in barley (27) and maize (19). SPS from a range of plant leaves is controlled by the Glc6P/Pi ratio (SC Huber, personal communication). Increased starch synthesis is accompanied by increased $Fru2,6P_2$ in maize (34), pea (2), and soybean (SC Huber, personal communication) and changes of SPS activity, although there may be differences between species (18, 25, 26). In soybean the changes are endogenous, while in maize the changes of SPS appear to be more dependent on the rate and duration of photosynthesis, and the export of sucrose from the leaf (18, 19).

Taken together, these studies suggest that photosynthetic sucrose synthesis is controlled by an interaction between the cytosolic Fru1,6Pase and SPS. As photosynthesis becomes faster, sucrose synthesis is favored by rising trioseP, falling $Fru2,6P_2$, a rising Glc6P/Pi ratio, and a light-activation of SPS. In addition, a decline in the measured SPS activity and an increase of $Fru2,6P_2$ lead to the restriction of sucrose synthesis, which allows starch to be rapidly accumulated.

These proposals are based on measurements of fluxes and metabolites in whole leaves. While $Fru2,6P_2$ is restricted to the cytosol (30), most other metabolites are present in the chloroplast stroma as well as in the cytosol. In previous studies, the overall metabolite contents have been interpreted on the assumption that the subcellular distribution of metabolites in leaves would resemble that in protoplasts (11, 12, 28, 29, 37), where the majority of the UDPglucose, hexoseP, and trioseP are in the cytosol. However, this assumption may not be justified, as protoplasts are an isolated system in which photosynthesis can be maintained for only a few minutes, and in which the normal path for sucrose export is missing.

Fractionation of frozen and lyophilized leaf homogenates by nonaqueous gradient centrifugation has been successfully applied for the determination of metabolite contents of the chloroplastic and extrachloroplastic fractionation of leaves (4, 13). This method has been further improved to achieve a partial separation of material deriving from the chloroplasts, cytosol, and vacuole

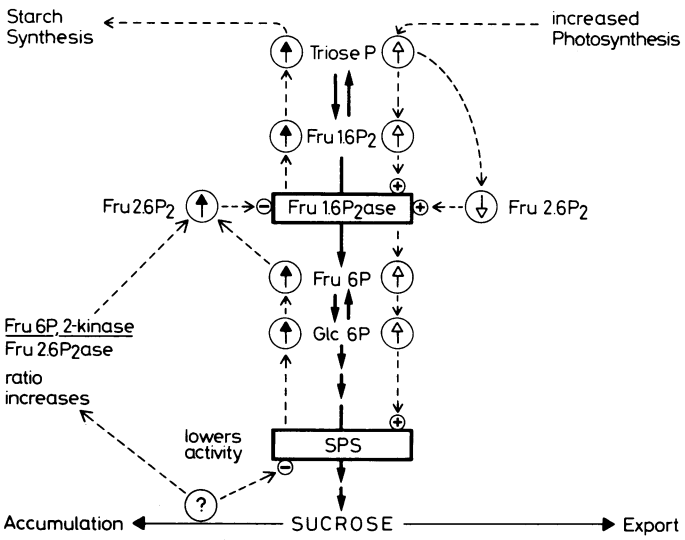

FIG. 1. Scheme for regulation of photosynthetic sucrose synthesis.

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[2] Abbreviations: trioseP, triosphosphate; $Fru1,6P_2$, fructose 1,6-bisphosphate; $Fru2,6P_2$, fructose 2,6-bisphosphate; $Ru1,5P_2$, ribulose 1,5-bisphosphate; Fru6P, fructose 6-phosphate; Glc6P, glucose 6-phosphate; UDPGlc, UDP-glucose; DHAP, dihydroxyacetone phosphate; PGA, 3-phosphoglycerate; $Fru1,6P_2$ase, fructose 1,6-bisphosphatase; SPS, sucrosephosphate synthase.

of spinach leaves (10). Leaf material is rapidly frozen in liquid N_2, ground, and lyophilized. The dried material is then ultrasonicated in a mixture of hexane/carbon tetrachloride and subjected to nonaqueous density gradient centrifugation, yielding a partial separation of material deriving from the chloroplast stroma, the cytosol, and the vacuoles. Marker enzymes and metabolites are assayed in 5 to 6 fractions, and the subcellular distribution of each metabolite is then estimated.

We have now applied this method to study how sucrose and the levels of phosphorylated intermediates vary in the subcellular compartments of leaves in conditions when sucrose metabolism is being altered. The results provide direct evident for our previous proposals about how the cytosolic Fru1,6Pase and SPS regulate sucrose synthesis in spinach leaves, and show that the phosphate translocator can exert a kinetic limitation *in vivo*.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea versus* Hybrid 424, Ferry Morse Seed Co., Mountain View, CA) was grown hydroponically in a climatized chamber with a 9 h light/15 h dark cycle (light intensity 400 $\mu E\ m^{-2}\ s^{-1}$) in air (360 $\mu l/L\ CO_2$), with a temperature of 22°C in the light and 16°C in the dark. Fully expanded leaves of 5- to 6-week-old plants were used.

Leaf material was frozen in liquid N_2, homogenized, and extracted in 10% $HClO_4$ as in Gerhardt and Heldt (10). Metabolites, sucrose, and starch were measured as in Stitt *et al.* (30, 37). For details of the nonaqueous subcellular fractionation technique, see Gerhardt and Heldt (10).

In experiments where only the total leaf metabolite content was measured, each sample contained 4 leaf discs (37 mm diameter) which were cut from 4 different plants and were immediately frozen in liquid N_2. At each time point, three samples were taken. For experiments where the subcellular distribution was studied, 5 to 6 spinach leaves were harvested at each time point from different plants, and were immediately frozen in liquid N_2. In some experiments, the leaves were subjected to 5 min in N_2 before transfer to liquid N_2. The results are the mean of three separate fractionations of this material. Similar results were obtained in fractionations carried out with separately harvested material.

RESULTS

Choice of Sampling Conditions. The experiments were carried out with spinach which was growing hydroponically in a 9 h light/15 h dark cycle (light 400 $\mu E \cdot m^{-2} \cdot s^{-1}$) in air. Photosynthesis of leaves from these plants became light- and CO_2-saturated at about 900 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 550 $\mu l/L\ CO_2$ (not shown). Samples were taken from plants at the end of the night, during illumination, and then after darkness at the times indicated. In separate experiments, samples were taken after 2 h in the light, or after illuminating leaves for a further 5 min in CO_2-free air to stop CO_2 fixation. Comparison of metabolites in the dark, in illuminated CO_2-free conditions, and in the light should reveal how sucrose synthesis is stimulated and inhibited in response to alterations of photosynthesis. Gradual changes occurring throughout 9 h in the light will show how metabolism is regulated during the day as partitioning is shifted in favor of starch later in the photoperiod.

Accumulation of Sucrose in the Vacuole. Figure 2 summarizes the changes of sucrose and starch in the spinach leaves. Both are almost exhausted at the end of the night (10, 30, 37). After the onset of illumination, there is a rapid accumulation of sucrose in the leaf, reflecting the difference between the rate at which sucrose is being synthesized and the rate at which it is being exported from the leaf. However, this accumulation of sucrose slows down later in the day and an increasing amount of photosynthate is retained in the chloroplast as starch. Extravacuolar sucrose increases during the first 30 min illumination, but there is no marked change during the remainder of the day, and it then declines through the night (Fig. 3). Thus, most of the sucrose accumulated in the leaf is found in the vacuolar compartment. There are also marked alterations of malate during the day and night. Most of the malate is stored in the vacuole (Fig. 3B). Unlike sucrose, where the vacuolar concentration does not exceed the concentration evaluated for the cytosol, the vacuolar concentration of malate appears to be much higher than that in the cytosol (10), reflecting an active transport of malate (23).

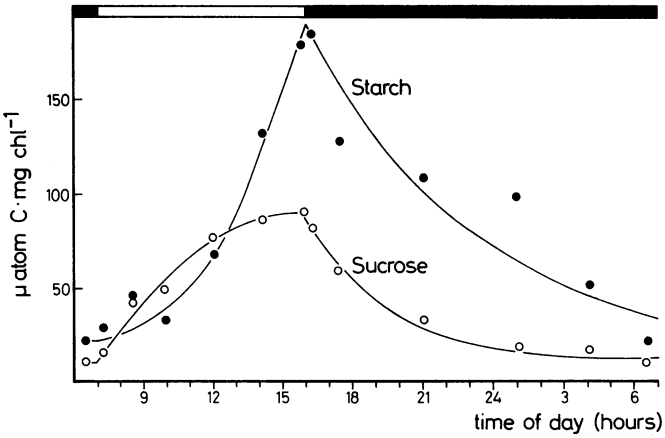

FIG. 2. Diurnal changes of starch and sucrose content in spinach leaves.

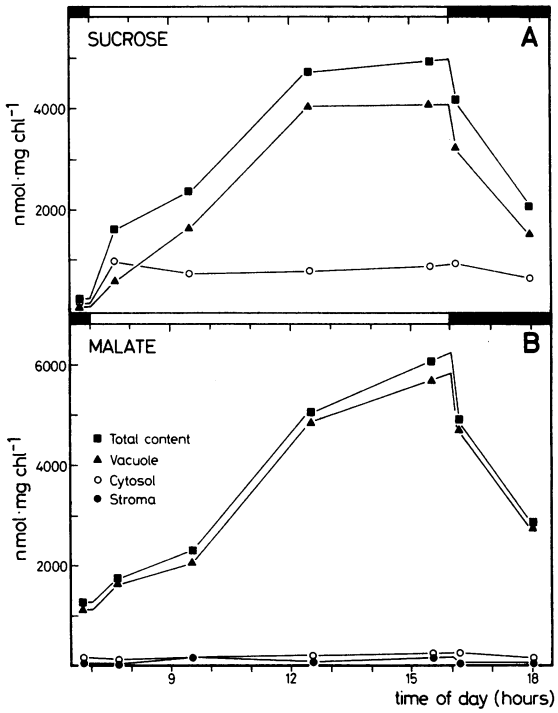

FIG. 3. Distribution of sucrose and malate in spinach leaves between the vacuole and extravacuolar compartments. A, Sucrose; B, malate.

Diurnal Alterations of Metabolites in the Stroma and Cytosol. Alterations of the total content of selected metabolites are shown in Figure 4. They can be divided into two groups, on the basis of the fluctuations in their overall content, as seen previously with leaves (1, 4, 29, 37) and protoplasts (29, 37). The metabolites in one group, which includes Ru1,5P_2, Fru1,6P_2, and DHAP, are hardly detectable in the dark and increase 20- to 100-fold in the light. In comparison with these large light/dark changes, only

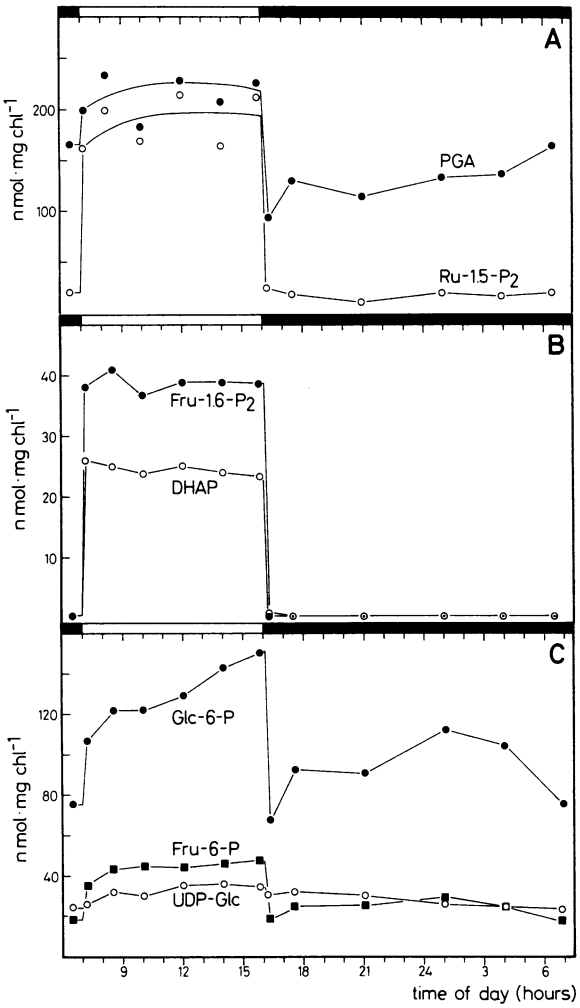

FIG. 4. Overall levels of metabolites during a 24 h cycle. A, PGA, Ru1,5P_2; B, DHAP, Fru1,6P_2; C, Glc6P, Fru6P, UDPGlc. Same experiment as described in Figure 2.

small changes occur during the day. The second group of metabolites includes PGA, Fru6P, Glc6P, and UDPGlc, which are present in the dark in considerable amounts and rise only slightly after illumination. However, these metabolites (except PGA) increase gradually throughout the illumination period.

The division of metabolites into groups become even clearer when their subcellular levels are determined (Fig. 5, Table I). With exception of PGA, the levels of other stromal metabolites (Fig. 5, A–C) are negligible in the dark, and increase sharply after illumination (Ru1,5P_2, Fru1,6P_2, DHAP, Fru6P, Glc6P). In individual experiments there was some variation between individual time points during the day, but these changes were small compared with the light/dark changes, and were not reproduced in other experiments (data not shown). Such variations as the higher Ru1,5P_2 after 2 h 30 min light or the lower stromal metabolites after 8 h 30 min shown in Figure 5 probably reflect tissue variability rather than a significant trend.

The cytosolic metabolites (Fig. 5, D–F) fall into two groups. DHAP and Fru1,6P_2 are present at negligible levels in the dark and increase markedly after illumination. In contrast, PGA, Fru6P, Glc6P, and UDPGlc do not increase greatly after the onset of illumination, and may even decrease. However, DHAP, Fru6P, Glc6P, and UDPGlc increase by 2- to 3-fold in the later phase of the illumination period.

Metabolite Levels in CO_2-Free Air. Table II compares metabolite levels in leaves killed after 2 h illumination in air, with those in leaves which were illuminated for a further 5 min in CO_2-free conditions before killing. The changes in the overall content resemble those previously reported for leaves in low CO_2 (1, 4, 32). In CO_2-free conditions Ru1,5P_2 increased about 2-fold to a level of about 400 nmol·mg^{-1} Chl, whereas all other stromal and cytosolic metabolites decreased. The decreases of stromal metabolites were particularly large, DHAP (8-fold), PGA (5-fold), Glc6P (3-fold), and Fru6P (4-fold). In the cytosol, PGA decreased markedly (5-fold), but other metabolites decreased only by 2-fold (Glc6P, Fru6P) or even less (DHAP, UDPGlc).

Alterations of Esterified Pi. Direct measurements of free Pi are complicated by the large amounts of Pi in the vacuoles of leaves. In preliminary experiments (not shown) we found that the bulk of the cellular Pi was present in the vacuole, and that accurate measurements of cytosolic or chloroplastic Pi would not be possible without further refinements of our method. However, the extent to which free Pi may change can be estimated by calculating how the total esterified P varies. Provided that movement of Pi across the tonoplast is not occurring, or is slow (9), an increase of esterified P would be accompanied by a decrease of free Pi.

In Table III the phosphate esterified in Ru1,5P_2, PGA, trioseP, Fru1,6P_2, Fru6P, and Glc6P is added together for each compartment. From the change of this sum between illumination and darkness (600–700 nmol·mg^{-1} Chl) and a stromal volume of 25 μl/mg Chl, a change of the free Pi in the order of 20 mM may be estimated for the stroma. In the cytosol the esterified phosphate decreases after darkening, but is not altered 40 min after the onset of illumination. In the course of the day, however, there is a gradual increase of esterified phosphate. The change of esterified phosphate (200 nmol·mg^{-1} Chl) during the day may represent a decrease of the cytosolic Pi by about 10 mM (cytosolic volume 20 μl·mg^{-1} Chl). Likewise, the change of the esterified phosphate in the cytosol after removing of CO_2 (100 nmol·mg^{-1} Chl) may indicate an increase of the cytosolic Pi by 5 mM.

Metabolite Gradients. As the level of many metabolites change in an independent manner in the stroma and the cytosol, their distribution between these compartments is not fixed, but depends on the conditions. The percentage of each metabolite found in the cytosol is compiled in Table IV. It should be noted that such estimates are not reliable in cases where a metabolite is largely confined to one compartment, as the correction for cross contamination (10) between fractions will then become very large in comparison to the amount of metabolite actually present in the other compartment. For example, the levels of Fru1,6P_2 estimated for the cytosol are only approximations.

As seen previously in protoplasts (11, 28, 29, 37), Ru1,5P_2 is restricted to the stroma, Fru1,6P_2 is mainly in the stroma, and UDPGlc is mainly in the cytosol. The distribution of Glc6P and Fru6P depended on conditions. In the dark, hexoseP were almost all in the cytosol. In the light, the stroma contained substantial hexoseP, but the proportion in the stroma declined later in the day. PGA is present in considerable amounts in the stroma and cytosol but more is present in the stroma in light (between 70 and 80% of the total) than in the dark (54–64%). The low levels of DHAP prevented its distribution being measured in the dark. In the light, most of the DHAP was found in the stroma at the beginning of the day, but the distribution changed in favor of the cytosol gradually during the day, and when leaves were transferred to CO_2-free conditions.

Free Energy Changes of Reactions. The determination of subcellular metabolite levels allows the evaluation of *in vivo* free energy changes of some stromal and cytosolic reactions (Table V). As the ΔG_0 values depend on Mg^{2+} concentration, pH, and other parameters, and since the ΔG_0 values are not determined under the conditions occurring in the cell, the results of our evaluation can be only a rough estimate. As expected from

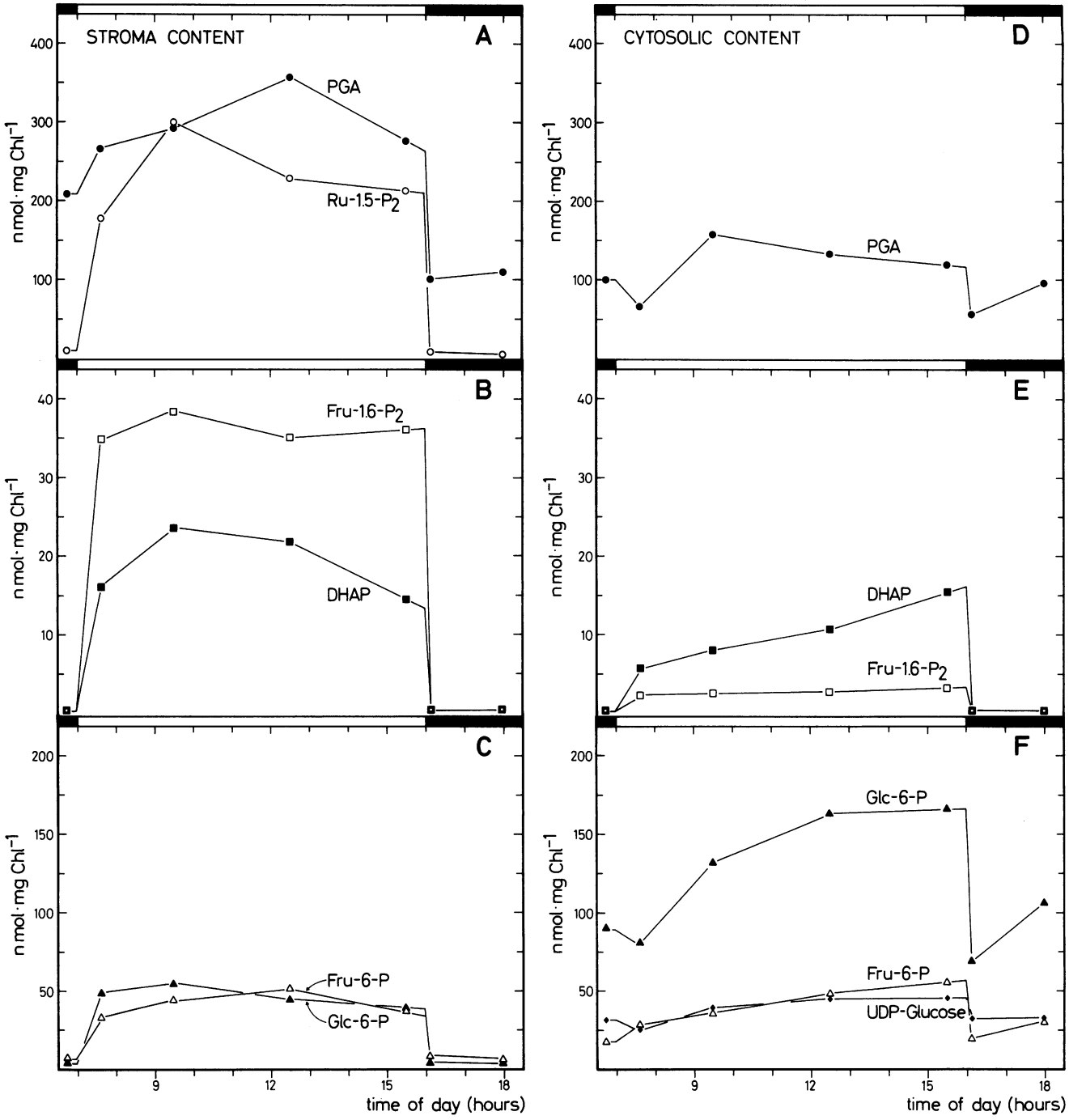

FIG. 5. Diurnal changes of subcellular metabolite levels in spinach leaves. Same experiment as described in Figure 3. Stromal metabolites: A, PGA, Ru1,5P_2; B, DHAP, Fru1,6P_2; C, Glc6P, Fru6P Cytosolic metabolites; D, PGA; E, DHAP, Fru1,6P_2; F, Glc6P, Fru6P.

previous work with whole leaves (5) and protoplasts (28), the reactions catalyzed by Ru1,5P_2 carboxylase and both the stromal and cytosolic Fru1,6P_2ase are displaced far from equilibrium, illustrating the role of these reactions in metabolic regulation. Whereas in the cytosol, the aldolase reaction appears to be near to equilibrium, for the stroma a considerable positive value for ΔG is obtained. Essentially similar results have been obtained in earlier measurements of metabolic levels in nonaqueously separated chloroplasts from leaves (4), with isolated chloroplasts (22), and with chloroplasts contained in protoplasts (28). The apparent displacement of this reaction in the direction opposite to the metabolic flux in the Calvin cycle cannot be explained at present. In the case of phosphoglucose isomerase, the Glc6P/Fru6P ratio (Tables I and II) in the cytosol is consistently found to be about 3 times higher than in the stroma (see also Ref. 4). The reaction appears to be in full equilibrium in the cytosol, but displaced from equilibrium in the stroma, as indicated from the negative ΔG value. It has been proposed that the stromal glucosephosphate isomerase has a rate limiting function in starch synthesis of leaves (5).

DISCUSSION

General Features of Subcellular Metabolite Levels. When CO_2 fixation is occurring, the levels of almost all stromal metabolites are markedly higher than when photosynthesis is prevented by

Table I. *Subcellular Metabolite Contents of Spinach Leaves under Air*

Data are from Figure 5.

	Light, 40 min		Light, 8½ h		Dark, 2 h	
	Stroma	Cytosol	Stroma	Cytosol	Stroma	Cytosol
	nmol/mg Chl					
Ru1,5P_2	183		217			
PGA	268	66	280	120	111	96
DHAP	16	6	14	16	2	2
Fru1,6P_2	35	2.3	36	4	1	1
Fru6P	34	27	37	55	6	30
Glc6P	48	82	39	167	4	106
UDPGlc	2	28	4	46	3	38
PGA/DHAP	17	11	20	8		
Glc6P/Fru6P	1.4	3.0	1.0	3.0	0.7	3.5

Table II. *Subcellular Metabolite Contents of Spinach Leaves Having Been Illuminated for 2 Hours under Air Only or with a Subsequent Illumination of 5 Minutes under N_2*

	Air		CO_2-free	
	Stroma	Cytosol	Stroma	Cytosol
	nmol/mg Chl			
Ru1,5P_2	249		420	
PGA	209	43	39	8
DHAP	16	5	2	3
Fru6P	27	32	6	15
Glc6P	26	106	9	58
UDPGlc	3	28	3	24
PGA/DHAP	13	9	20	3
Glc6P/Fru6P	1.0	4.3	1.5	3.9

Table III. *Summed Phosphate Esterified in Intermediates in the Chloroplast Stroma and Cytosol*

Calculated from the data of Figure 5.

Conditions	P Ester	
	Stroma	Cytosol
	nmol·mg^{-1} Chl	
A. 15 h dark	219	240
40 min light	802	213
8 h 30 min light	873	413
10 min dark	115	178
2 h dark	121	268
B. 2 h light	820	240
2 h light, 5 min N_2, CO_2-free	901	108

removal of CO_2 or by darkness. These changes were observed in all our experiments. During the day, however, we have not yet been able to establish clear trends in the stroma as the small fluctuations of metabolites found in one experiment were not reproducible in others, and may just reflect variability in the tissue sampled. The metabolite pools in the cytosol show a different pattern. Thus, while some cytosolic metabolites such as DHAP and Fru1,6P_2 change greatly when photosynthesis begins, others, including hexoseP and UDPGlc, do not alter much during such transitions. However, all the cytosolic metabolite levels show a 2- to 3-fold gradual rise throughout the day. The different behavior of these subcellular metabolite pools will be discussed in terms of the regulation of sucrose synthesis, metabolism in the stroma, and transport processes.

Activation of Cytosolic Fru1,6Pase in Response to Photosynthesis. When photosynthesis is stopped in CO_2-free conditions, there is also a decrease of DHAP in the cytosol (Table II) but the change is relatively small. This resembles earlier studies with protoplasts, where the Fru1,6Pase was inactivated by relatively small changes of DHAP in the cytosol (29). Such observations have been incorporated into a model which predicts that the cytosolic Fru1,6Pase remains inactive until a 'threshold' level of DHAP is reached, but is sharply activated once this threshold is exceeded (16, 35, 40).

The data shown in Table II now allow this threshold concentration in the cytosol of leaves to be estimated. Whereas a cytosolic trioseP content of 5 nmol/mg Chl is found in leaves under air, which carry out photosynthesis and sucrose synthesis, this value decreases to 3 nmol/mg Chl when photosynthesis is brought to a stop because of lack of CO_2. The threshold value of trioseP required for sucrose synthesis would therefore have to be in the range between these two values. This would be equivalent to a cytosolic concentration of about 0.25 mM DHAP (assuming a cytosolic volume of 15–20 $\mu l \cdot mg^{-1}$ Chl), and is a factor of 3 below the predicted concentrations of DHAP needed to activate Fru1,6Pase *in vitro* in simulation experiments (35, 40). The discrepancy is probably due to the assumptions involved in deriving our model. For example, it was assumed that all the Fru2,6P_2 *in vivo* is free, but since Fru2,6P_2 is present at low (μM) concentrations and binds with high affinity on target enzymes, it is likely that a significant proportion of the Fru2,6P_2 will actually be bound *in vivo*. For this reason, the *in vitro* simulation probably overestimated the concentration of free Fru2,6P_2, and therefore overestimates how much DHAP is required to allow activity of the Fru1,6Pase.

Activation of Sucrose Phosphate Synthase in Response to Photosynthesis. SPS is activated by Glc6P, and inhibited by Pi (6, 18, 19). It was therefore suggested that a rising Glc6P/Pi ratio would stimulate SPS as photosynthesis increases and hexoseP is produced (38). Our measurements confirm that Glc6P decreases 2- to 3-fold in the cytosol when photosynthesis is stopped by darkening leaves or excluding CO_2, and suggest that cytosolic Pi may alter in a reciprocal manner. The substantial levels of hexoseP and UDPGlc which remain show that SPS activity has been inhibited by regulation, and not just by depletion of substrates. These, and similar results with protoplasts (29), provide clear evidence that SPS is regulated by metabolites *in vivo* as the rate of photosynthesis varies. The results have been incorporated into a model (40) suggesting that SPS resembles the cytosolic Fru1,6Pase, being first activated once a threshold level of hexoseP is reached.

However, our results also demonstrate that additional regulatory mechanisms are needed *in vivo*. Upon illumination at the beginning of the day, Glc6P and Pi do not change appreciably,

Table IV. *Distribution of Metabolites in the Cytosol, Expressed as Percentage of the Total*
Calculated from the data of Figure 5.

	Percent of Metabolite in the Cytosol					
	PGA	DHAP	Fru1,6P$_2$	Fru6P	Glc6P	UDPGlc
A. 15 h dark	32	ND[a]	ND	71	96	90
40 min light	20	27	7	45	63	90
2 h 30 min light	35	25	7	44	71	90
5 h 30 min light	27	34	7	47	79	90
8 h 30 min light	30	51	9	59	82	90
15 min dark	36	ND	ND	70	94	90
2 h dark	46	ND	ND	83	96	90
B. 2 h light	17	24	ND	54	80	90
2 h light, 5 min N_2, CO_2-free	20	60	46	71	87	90

[a] ND, not detectable.

Table V. *Calculated Values for Free Energy Changes of Stromal and Cytosolic Enzymic Reactions in Spinach Leaves during Photosynthesis under Air*
Average values from the data in Figure 5.

Enzyme	Standard Free Energy ΔG_0	Free Energy Change *in Vivo* ΔG	
		Stromal reaction	Cytosolic reaction
	KJ/mol		
1. Ru1,5P$_2$ carboxylase	−35.3	−11.5[a]	
2. Aldolase	−23.1	+4.6[b]	+0.8
3. Frul,6Pase	−16.8	−28.2[c]	−32.3[c]
4. Phosphoglucose isomerase	−3.0	−2.7	0.1

[a] Assumed (CO_2) = 10 μM. [b] For the case that trioseP are in trioseP isomerase equilibrium. [c] Assumed (P_i) = 10 mM.

Table VI. *Concentration Gradients for DHAP Across the Chloroplast Envelope Membrane*
Estimated from the results of Figure 5, assuming a stromal volume of 25 μl and a cytosolic volume of 20 μl.

Conditions	$\frac{(DHAP)_{stroma}}{(DHAP)_{cytosol}}$	Concentration Difference between Stroma and Cytosol
	ratio	*mM*
A. 40 min light	2.1	+0.34
2 h 30 min light	2.4	+0.56
5 h 30 min light	1.5	+0.29
8 h 30 min light	0.7	−0.24
B. 2 h light	2.6	+0.39
2 h light + 5 min light (N_2, CO_2-free)	0.5	−0.07

and the levels of the substrates of SPS hardly rise (Fru6P) or even decrease (UDPGlc). Similar results were obtained after illuminating spinach protoplasts (28) and led to the conclusion that a light-dependent activation of SPS was required. SPS activity actually increases up to 2-fold after illuminating spinach leaves (40) and similar light-dependent changes have been reported for barley (27) and maize (19). It is not known what is responsible for these light-dependent alterations of SPS activity, but our results indicate that they interact with the control by metabolites and allow the conversion of hexoseP to sucrose to be activated without this necessarily needing an increase in the cytosolic hexoseP pool.

Export and Storage of Sucrose. Sucrose is exported in the phloem, but some sucrose is also accumulated in the vacuole of spinach leaves, as has also been shown for barley protoplasts (20). Accumulation of sucrose declines as the day progresses, suggesting that the rate of sucrose synthesis has been modified (see below) so that it matches export more closely and more photosynthate is accumulated as starch. To understand these changes in the export, storage, and synthesis of sucrose, we need to know how the sucrose concentration in the cytosol is varying. Our method reveals how the extravacuolar sucrose varies in leaves. This probably includes not just the sucrose in the cytosol of photosynthetic cells but also the sucrose in vascular tissue and any sucrose in the cell wall (10). At the beginning of the day, the extravacuolar sucrose rapidly increases about 5-fold and remains at about this level during the remainder of the day. An increase in one or more of the extravacuolar pools could therefore contribute to stimulating export of sucrose out of the leaf at the beginning of the day.

The maximal concentration of sucrose in the cytosol can be estimated as about 10 mM in the dark, and 40 to 50 mM in the light (Fig. 6), assuming that all the extravacuolar sucrose is restricted to the cytosol of mesophyll cells. The highest concentration reached in the vacuole would be about 30 mM (assuming a vacuolar volume of 150 $\mu l \cdot mg^{-1}$ Chl volume), so uptake into the vacuole could be passive, if the cytosol accounts for most of the extravacuolar sucrose. This agrees with studies of isolated

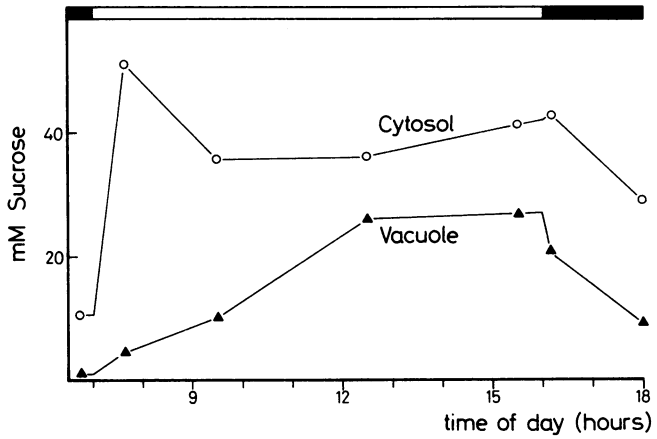

FIG. 6. Estimated concentrations of sucrose in the vacuole and extravacuolar compartments of spinach leaves (data from Fig. 3).

barley leaf vacuoles, showing that they possess a passive sucrose transport system (21).

Our results do not provide evidence for an increase of sucrose in the cytosol during the day as storage in the vacuole is decreasing and the partitioning of photosynthate is being altered in favor of starch. These results do not prove that the cytosolic level remains unchanged as there may be multiple pools of sucrose in the leaf, but any significant increase in the cytosol would require that sucrose decreases in the cell wall or in vascular tissue during the day, which seems somewhat unlikely. Clearly, it remains an open question, whether alterations of sucrose are directly involved in controlling sucrose synthesis and partitioning, or whether other regulatory mechanisms must be postulated, which allow sucrose storage, export, and metabolism to be controlled independently of the cytosolic sucrose level. It might be noted that 50 mM sucrose has no direct effect on SPS, nor on the activity of enzymes which synthesize or degrade $Fru2,6P_2$ (M Stitt, unpublished data). A correlation between sucrose accumulation and inhibition of sucrose synthesis does not necessarily mean that the accumulation of sucrose is directly causing the rate of sucrose synthesis to be altered. If export of sucrose were remaining constant, then a change in the rate of sucrose accumulation could equally result from a change in the rate of sucrose synthesis.

Coordinate Inhibition of SPS and the Cytosolic Fru1,6Pase during the Day. While the ultimate cause remains unclear, the results in Figure 5 provide clear evidence that sucrose synthesis is increasingly restricted in the course of the day as an increasing proportion of the photosynthate is being diverted toward starch, but the levels of metabolites rise in the cytosol. The accumulation of its substrates (UDPGlc and Fru6P) shows that SPS activity has been restricted, while the increase of DHAP and $Fru1,6P_2$ reveals that the activity of the cytosolic Fru1,6Pase has been decreased. What is immediately responsible for the lower activity of these enzymes?

For SPS, the concentrations of substrates (Fru6P and UDPGlc) and of activator (Glc6P) increases during the photoperiod, while the concentration of the inhibitor, Pi, may even decrease, which would be expected to even increase SPS activity later in the photoperiod (see above). Therefore, other regulatory mechanisms must be acting to compensate for this regulation by metabolites. This may involve changes in the amount of SPS activity in the leaf, or in the activation state of SPS. It is known that the activity of SPS, which can be extracted from leaves, varies during the day in many species (18, 19, 26). Recently, we have found a gradual 20 to 40% decline in the activity of SPS between 30 min and 8.5 h in the light (40), which is linked with a change in the kinetic properties, so the enzyme becomes more sensitive to inhibition by Pi (15). Further studies are needed to clarify whether these changes in the amount of activity are due to protein modification, or protein turnover. The restriction of cytosolic Fru1,6Pase activity can be explained by the gradual increase of $Fru2,6P_2$ in the cytosol during the day (30, 35). But why does $Fru2,6P_2$ decrease at the beginning of the day and increase again later?

Regulation of the $Fru2,6P_2$ Concentration. $Fru2,6P_2$ is synthesized and degraded by specific enzymes, called Fru6P,2-kinase and Fru2,6Pase, respectively, whose activity is regulated by metabolites (3, 31, 36). Our results support the previous proposal that regulation of Fru6P,2-kinase by DHAP and PGA allows $Fru2,6P_2$ to be decreased as photosynthesis increases (31, 32). DHAP increases markedly when leaves are illuminated (Fig. 5E), which will decrease $Fru2,6P_2$. The inhibition of Fru6P,2-kinase by DHAP is particularly effective when Pi is high and Fru6P is low (36) in leaves at the beginning of the day. When photosynthesis is inhibited by low CO_2, the large decrease of PGA in the cytosol (Table I) will inhibit Fru6P,2-kinase, as previously suggested from measurements in whole leaves (32).

The gradual increase of $Fru2,6P_2$ in leaves during the day was previously interpreted as a consequence of the decreased SPS activity. When SPS is inhibited there is an accumulation of Fru6P, which should stimulate the synthesis and inhibit the breakdown of $Fru2,6P_2$ (31, 33). The measurements in Figure 4 confirm that Fru6P rises 2-fold in the cytosol during the day, but our earlier explanation has to be extended, since DHAP also increases 2- to 3-fold. This increase of DHAP would be expected to inhibit the Fru6P,2-kinase and prevent the increases of $Fru2,6P_2$. This problem was previously not apparent, because measurements in whole leaves did not reveal the marked accumulation of DHAP in the cytosol in the course of the day. Two recent findings may provide an answer. As inhibition of Fru6P,2-kinase by DHAP becomes weaker as Pi is decreased (36), the declining cytosolic Pi during the day (see above) will weaken the effectiveness of DHAP as an inhibitor, partly counteracting the increased DHAP concentration. However, the results of Figure 5 led us to reinvestigate whether the concentration of $Fru2,6P_2$ is also controlled by other mechanisms in leaves, apart from the previously characterized control by metabolites. We have recently found that the rise of $Fru2,6P_2$ during the day is accompanied by a 2-fold increase of the Fru6P,2-kinase: Fru2,6Pase ratio (39, 40). Further studies are needed to show what is causing these changes, which will contribute to the rise of $Fru2,6P_2$ as partitioning into starch is increased.

Kinetic Limitation by the Phosphate Translocator. The discussion so far shows how sucrose synthesis is activated as the chloroplast utilizes Pi and provides trioseP, and how the cytosol can restrict sucrose synthesis and decrease the demand for trioseP so that more starch is accumulated in the chloroplast. This transport of trioseP and Pi between the stroma and cytosol is catalyzed via the phosphate translocator (8). As it transports its substrates in both directions in a passive manner, the rate and direction of the exchange of trioseP and Pi depends upon their concentrations in the two compartments (7, 8), and alterations in fluxes and concentrations in one compartment will be transmitted to the other.

With isolated chloroplasts, the phosphate translocator shows an excess of activity and to attain optimal rates of photosynthesis it is necessary to restrict the activity of the translocator by using low Pi concentrations in the medium, or by inhibiting the translocator partially by adding PPi or pyridoxal P (8). However, isolated chloroplasts provide a special case because only one potential substrate (Pi) is present outside the chloroplast. *In vivo*, in the cytosol there are also considerable pools of the other, competing, substrates DHAP and 3PGA. *In vivo*, a considerable portion of the translocator capacity will be catalyzing homologous exchanges, transfer of Pi and PGA, or even a reversed uptake of trioseP in exchange for Pi. The translocator might still exert a kinetic limitation *in vivo*, even though the maximal activity of the translocator exceeds the rate of photosynthesis. Any such kinetic limitation will mean that substantial concentration gradients of trioseP, PGA, and Pi could develop across the envelope membrane during rapid photosynthesis, and would disappear rapidly when the rate of photosynthesis is decreased in the dark or in low CO_2. Inspection of Table IV confirms there is variation in the distribution of DHAP and PGA, depending on the conditions.

Interpretation of these data is complicated, because the transport process is itself affected by illumination. In isolated chloroplasts, illumination leads to an alkalization of the stroma by 0.5 to 1.0 pH units (14), and recent studies on CO_2 absorption by leaves have been interpreted as showing that similar pH changes are also occurring in the chloroplasts *in vivo* (24). The resulting pH gradient across the envelope influences transport (7), leading to retention of PGA in the stroma. This occurs because of pH

dependent changes of the kinetic properties of the phosphate translocator (7), and because in alkaline conditions most of the PGA is present as PGA^{3-}. PGA is transported by the translocator, like the other substrates, as divalent anion. Consequently, in illuminated chloroplast suspensions the PGA/trioseP ratio in the medium is usually much lower than in the stroma (8). In analogy we would expect a much higher stromal PGA/trioseP ratio in the stroma than in the cytosol of illuminated leaves. In fact, PGA/trioseP ratios in the cytosol and stroma differ only by a factor of 2.5 or less when leaves are illuminated in the air (Table I).

When the leaves are illuminated in the absence of CO_2 to prevent CO_2 fixation (Table II), however, the PGA/trioseP ratio in the cytosol is about 7 times lower than that in the stroma. Thus, the light-dependent alkalization of the stroma is revealed best in low CO_2, when the photosynthetic fluxes are slowed to allow PGA and trioseP to distribute at nearer to their expected equilibrium. In air, however, this asymmetrical distribution is less pronounced, suggesting that the phosphate translocator is kinetically limiting and the presence of a pH gradient is masked. An asymmetric distribution of PGA and trioseP was previously found during induction in spinach protoplasts, when photosynthetic fluxes would be lower (28). Also recalculation of published results for wheat protoplasts (29) shows that the $(PGA/trioseP)_{stroma}$: $(PGA/trioseP)_{cytosol}$ quotient, which was low (2–4) in the light, increased markedly 10 s after darkening (20–40) and decreased again during the next 60 s in the dark (back to 2–4). The high quotient immediately after darkening could reflect the light-dependent alkalinization, which then decays as the period in the dark is extended.

The consequence of a kinetic limitation of transport for sucrose synthesis is illustrated in Table VI. There is a gradient of DHAP from the stroma to the cytosol during rapid photosynthesis, which disappears in CO_2-free air, as expected if this gradient is required to allow the rapid fluxes during photosynthesis and collapses when the fluxes are slowed or stopped. There is also a gradual redistribution of DHAP in favor of the cytosol throughout the day, which can be explained in terms of a shifting relation between the supply of DHAP from photosynthesis and its consumption in the cytosol as sucrose synthesis is restricted. At the beginning of the day, the cytosolic Fru1,6Pase has a high demand for substrate and trioseP are rapidly removed and converted to sucrose in the presence of low metabolite levels and high Pi. A large concentration gradient of trioseP is needed to allow rapid transport in the presence of high Pi, and an active cytosolic Fru1,6Pase may even provide the driving force for this exchange by removing trioseP in the cytosol. Later in the day, however, the activity of SPS and the cytosolic Fru1,6Pase has been inhibited (see above), so fluxes are decreasing while metabolites accumulate and Pi may decline. A smaller gradient of trioseP is needed, and could even become negative if the decreasing rate of sucrose synthesis alters the relation between Pi consumption in the stroma and Pi release in the cytosol so far, that a large concentration gradient of Pi develops in the direction of the stroma.

Stromal Metabolites and Photosynthesis. The large changes of $Ru1,5P_2$ and metabolites involved in the regenerative phase of the Calvin cyle may contribute to the strong on-off regulation of the Calvin cycle in response to light, as a large change of the substrate concentration will reinforce the light-dependent activation of stromal enzymes by thioredoxin, and changes of pH and Mg^{2+}. Our measurements do not reveal any consistent trend in stromal metabolites during the day. This is surprising, because an increase of PGA would be expected in conditions where sucrose synthesis is being inhibited and starch is accumulating. Our failure to observe this change, however, could be due to variation between plants, because the experiments were carried out in limiting light and CO_2 when the alterations of Pi may not have been so large that large alterations of PGA occurred in a given leaf. As will be shown elsewhere, Pi can become a limiting factor during photosynthesis in saturating light and CO_2, and studies in such conditions may provide clearer information about how chloroplast metabolites respond *in vivo* to alteration in Pi availability.

CONCLUSIONS

These results confirm that a coordinate control of the cytosolic Fru1,6Pase and SPS, exerted by metabolites, $Fru2,6P_2$, and alterations in the amount of enzyme activity, allow sucrose synthesis to be regulated in relation to the availability of photosynthesis, and also allow photosynthate partitioning to be modified. They also reveal how the phosphate translocator can exert a kinetic limitation during photosynthesis *in situ*. Finally, they raise the question, what causes the alterations of SPS and Fru6P,2-kinase activity which are instrumental in restricting sucrose synthesis so that more starch is accumulated. More work is needed to clarify the mechanisms underlying these changes, as well as the signal which triggers these mechanisms.

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