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Control of Photosynthetic Sucrose Synthesis by Fructose 2,6-Bisphosphate[1]

I. COORDINATION OF CO_2 FIXATION AND SUCROSE SYNTHESIS

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ABSTRACT

A mechanism is proposed for a feed-forward control of photosynthetic sucrose synthesis, which allows withdrawal of carbon from the chloroplast for sucrose synthesis to be coordinated with the rate of carbon fixation. (a) Decreasing the rate of photosynthesis of spinach (*Spinacia oleracea*, U.S. hybrid 424) leaf discs by limiting light intensities or CO_2 concentrations leads to a 2-to 4-fold increase in fructose 2,6-bisphosphate. (b) This increase can be accounted for by lower concentrations of metabolites which inhibit the synthesis of fructose 2,6-bisphosphate, such as dihydroxyacetone phosphate and 3-phosphoglycerate. (c) Thus, as photosynthesis decreases, lower levels of dihydroxyacetone phosphate should inhibit the cytosolic fructose bisphosphatase via simultaneously lowering the concentration of the substrate fructose 1,6-bisphosphate, and raising the concentration of the inhibitor fructose 2,6-bisphosphate.

The aim of the experiments described in this and accompanying papers (13, 21) was to show how the signal metabolite Fru 2,6-P_2[2] provides one way of controlling photosynthetic sucrose synthesis. Sucrose is the major endproduct of photosynthesis in most leaves, as well as the form in which carbohydrate is exported to the rest of the plant, but the processes controlling its formation from the immediate products of photosynthesis are not understood. A control of sucrose synthesis is essential, however, not only so that the production of sucrose can be adjusted to different conditions or requirements, but also to maintain the efficient functioning of metabolism in the leaf.

Triose P produced by the chloroplasts during photosynthesis are the starting point for sucrose synthesis, and represent an important branch point in photosynthetic metabolism. Most of the triose P are retained in the Calvin cycle to regenerate RuBP which is the acceptor for further CO_2 fixation. However, surplus triose P can be converted into the endproducts of photosynthesis, so that most of the surplus is usually transferred into the cytosol and converted to sucrose. The rate of sucrose synthesis must be matched to the rate at which CO_2 can be fixed and reduced to triose P. It is known that photosynthesis by isolated chloroplasts is prevented by high Pi concentrations in the medium, as rapid entry of Pi into the chloroplast in exchange for triose P depletes the concentration of metabolites in the stroma. This inhibits the Calvin cycle enzymes so that no more RuBP can be regenerated (22). A similar situation will arise *in vivo* if the rate of sucrose synthesis exceeds the rate of photosynthesis. During sucrose synthesis, Pi is released from triose P and reenters the chloroplast in exchange for further triose P; if this occurs faster than the rate at which triose P can be produced, then the stromal metabolites will decrease and photosynthesis will be inhibited. In fact, studies with wheat protoplasts have shown that sucrose synthesis and photosynthesis are coordinated *in vivo*, and that use of triose P for synthesis of sucrose is very rapidly inhibited when the rate of photosynthesis decreases (13). This allows substantial levels of metabolites to be maintained in the stroma even when photosynthesis is very slow.

Triose P are also a branch point in the partitioning of photosynthate between sucrose and starch. During rapid photosynthesis, sucrose will tend to accumulate in the leaf if the rate of synthesis is higher than the rate of export. The extent to which sucrose accumulates seems to depend on the species (7, 10, 19) but usually a point is reached when accumulation of sucrose slows down or stops, and is replaced by accumulation of starch in the chloroplast (7, 10). This implies that conversion of triose P to sucrose in the cytosol is being restricted, so that some of the surplus triose P can be retained in the chloroplast for converison to starch.

Although it is clear that sucrose synthesis must be regulated in leaves, it is not known how this regulation is achieved, either to coordinate the rate of sucrose synthesis with the ability of the Calvin cycle to provide triose P during photosynthesis in limiting conditions, or to restrict sucrose synthesis during rapid photosynthesis in order to divert a portion of the available triose P into starch. The first irreversible reaction during the conversion of triose P to sucrose in the cytosol is catalyzed by the cytosolic FBPase. Although this enzyme should be regulated in order to control withdrawal of carbon from the chloroplast, studies of the partially purified enzyme revealed a very low K_m for its substrate and only weak inhibition by high concentrations of AMP or Pi (8, 11, 23).

Subsequently, it was found that the cytosolic FBPase is inhibited by Fru 2,6-P_2 (2,16), a new signal metabolite first discovered in liver (12). At low concentrations, Fru 2,6-P_2 decreases the substrate affinity of the leaf cytosol FBPase, and increases the sensitivity to inhibition by AMP (2,16). Fru 2,6-P_2 is present in the cytosol (2, 19) of spinach leaves at concentrations of the order of 1 to 15 μM (16, 19), where it is synthesized and degraded by specific enzymes, the Fru 2,6-P_2 kinase (3) and Fru 2,6-bisphosphatase (4). As will be described elsewhere in detail, the activity of these enzymes can be regulated by metabolites like

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[2] Abbreviations: Fru 2,6-P_2, fructose 2,6-bisphosphate; DHAP, dihydroxyacetone phosphate; FBPase, fructose 1,6-bisphosphatase; Fru 1,6-P_2, fructose 1,6-bisphosphate; Glc 6-P, glucose 6-phosphate; PGA, 3-phosphoglycerate; RuBP, ribulose 1,5-bisphosphate.

PGA, DHAP, Fru 6-P and Pi (Stitt, Cseke, and Buchanan, in preparation).

In experiments presented in this and following articles (13, 21), we have measured the levels of Fru 2,6-P_2 and other metabolites in spinach leaves in conditions when the rate of sucrose synthesis is varied, either by altering the rate of photosynthesis, or by allowing sucrose to accumulate so that more of the photosynthate is diverted to starch. Comparison of the results with the properties of the cytosolic FBPase (13) and with the properties of the enzymes which synthesize and degrade Fru 2,6-P_2 (3, 4, 20) shows that the Fru 2,6-P_2 level in leaves changes in response to perturbations in effector metabolites, and that these alterations of Fru 2,6-P_2 play an important role in controlling sucrose synthesis. The present article shows how changes of the Fru 2,6-P_2 concentration in response to alterations in the supply of triose P allow the activity of the cytosolic FBPase and the rate of sucrose synthesis to be adjusted to the rate of carbon fixation.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* U.S. hybrid 424, Ferry Morse Seed Co.) was grown in water culture in a 9-h light/15-h dark cycle as in Lilley and Walker (14). The light intensity was provided by 150-w tungsten and 250-w mercury lamps. The temperature was 22°C in the light and 18°C in the dark. Before all experiments, the spinach plants were darkened for 15 to 18 h.

For experiments on leaves, samples were taken directly from leaves on the plant and plunged immediately into liquid N_2. In experiments with leaf discs, leaves were removed from the plant and kept in water-saturated air in the dark for 1 to 3 h. Leaf discs (diameter 35 mm) were cut out immediately before the experiment, and divided into quarters which were incubated in an O_2 electrode (5) at 20°C. Samples were taken by rapidly opening the electrode, removing leaf material and plunging it into liquid N_2. Illumination was provided by a projector and the intensity was varied by altering the applied voltage. Differing CO_2 concentrations were provided by varying the pH and molarity of a $K_2CO_3/KHCO_3$ buffer. Due to the diffusion pathways, the absolute CO_2 concentration in the electrode is not known (Walker, personal communication) and conditions were empirically selected to achieve different rates of O_2 evolution by the leaf material.

Fru 2,6-P_2 was extracted and assayed as in Stitt *et al.* (19). HexoseP, triose P, UDP glucose, and FBP were measured as in Stitt *et al.* (18) in the extracts used to measure Fru 2,6-P_2. Chl was measured as in Stitt *et al.* (19).

RESULTS AND DISCUSSION

On the Approach. In earlier experiments with spinach plants (19), it was found that the Fru 2,6-P_2 level decreased during the initial 10 to 15 min of illumination, which should stimulate sucrose synthesis by relieving the inhibition of the cytosolic FBPase. To investigate in more detail the possibility that Fru 2,6-P_2 acts as a link between photosynthesis and sucrose synthesis, we studied the effect of a range of different light intensities and CO_2 concentrations on Fru 2,6-P_2 levels. Leaf discs were incubated in a leaf O_2 electrode (5) so that photosynthesis could be monitored in defined conditions before freezing the leaf material in liquid N_2, and analyzing it for Fru 2,6-P_2 and other phosphorylated intermediates.

Two types of experiments were carried out. In one approach, leaf discs were illuminated in saturating light and CO_2 to obtain maximal rates of photosynthesis, before suddenly lowering the CO_2 concentration or the light intensity. This allowed the response of Fru 2,6-P_2 to be studied during a rapid transition between different conditions. In a second approach, leaf discs were allowed to carry out photosynthesis at several constant light intensities or CO_2 concentrations for 10 min to study the relation between the rate of photosynthesis, the level of Fru 2,6-P_2, and the amounts of other metabolites in the tissue in a wider range of conditions.

Fru 2,6-P_2 Levels and Photosynthesis. During a transition from darkness to light, and back to darkness again, the Fru 2,6-P_2 decreased by more than 60% in the light, and increased again in the dark, although the original level before illumination was not reached within 10 min (Fig. 1A.). When the CO_2 concentration was lowered, instead of removing the light, a 70% inhibition of photosynthesis was accompanied by a 50% increase in the Fru 2,6-P_2 level (Table I). In the experiments of Figures 2 and 3, the rate of photosynthesis was adjusted by varying the light intensity or CO_2 concentration, respectively. In all cases, steady rates of photosynthesis were achieved within 10 min, before the leaf material was quenched. Independent of the treatment, the level of Fru 2,6-P_2 rose as photosynthesis rates became lower. This suggests there is indeed an inverse relation between Fru 2,6-P_2 and photosynthesis over the entire range of conditions between rapid photosynthesis and total inhibition of photosynthesis.

The significance of the altered Fru 2,6-P_2 will depend partly upon how quickly it changes in response to an altered rate of photosynthesis. Our experiments show that these changes can be quite rapid. After turning the light on (Fig. 1A), the Fru 2,6-P_2 content decreased rapidly 2 to 5 min later, and this actually represents a minimal estimate of the rate at which Fru 2,6-P_2 alters in response to the metabolite status of the leaf, as the Fru 2,6-P_2 level alters in response to changes in the concentrations of metabolite effectors (see below), and these themselves only alter over a period of minutes. In spinach leaves, 2-fold alterations of Fru 2,6-P_2 was also found within a timespan of 5 min after illuminating or darkening leaves (19).

Metabolite Levels in Leaves. To investigate what might be responsible for the alterations in Fru 2,6-P_2 level, as well as to assess what contribution Fru 2,6-P_2 may make to control of sucrose synthesis compared with other regulatory mechanisms, we also measured various other metabolites in these experiments. Although only the total metabolite content of the leaf material was measured in these experiments, marked alterations do allow some suggestions to be made as to the levels in the different compartments as DHAP, Glc 6-P, and UDPGlc are primarily located in the cytosol, while PGA and especially Fru 1,6-P_2 are mainly located in the stroma (9, 15, 18).

The level of hexose P declined gradually as photosynthesis was decreased by lower light or CO_2 (Figs. 2B and 3B) but substantial amounts still remained at low rates of photosynthesis, or even when photosynthesis was suddenly stopped by darkness (Fig. 1B). The level of triose P also declined as light or CO_2 became limiting, and was very low in the dark (Figs. 1B, 2B, and 3B). The PGA content was slightly lower in the dark or when photosynthesis was low, especially when CO_2 was limiting. In contrast, the Fru 1,6-P_2 level remained very constant in all conditions, except in the dark when negligible Fru 1,6-P_2 was found.

These alterations in overall metabolite levels in spinach leaves are very similar to those found in more detailed studies of subcellular metabolite levels in wheat protoplasts carrying out photosynthesis in various CO_2 concentrations and light intensities (18). In these experiments, the cytosolic hexose P and triose P remained relatively high when photosynthesis rates were low, and the Fru 1,6-P_2 even rose. It was concluded that relatively small fluctuations of the cytosolic metabolites were sufficient to lead to inhibition of sucrose P synthase and the cytosolic FBPase, so that considerable concentrations of metabolites in the stroma, such as Fru 1,6-P_2, could be maintained even when photosynthesis was up to 90% inhibited. The overall agreement between the results in leaves and protoplasts suggests that the conclusions reached in the simplified model system of protoplasts are valid

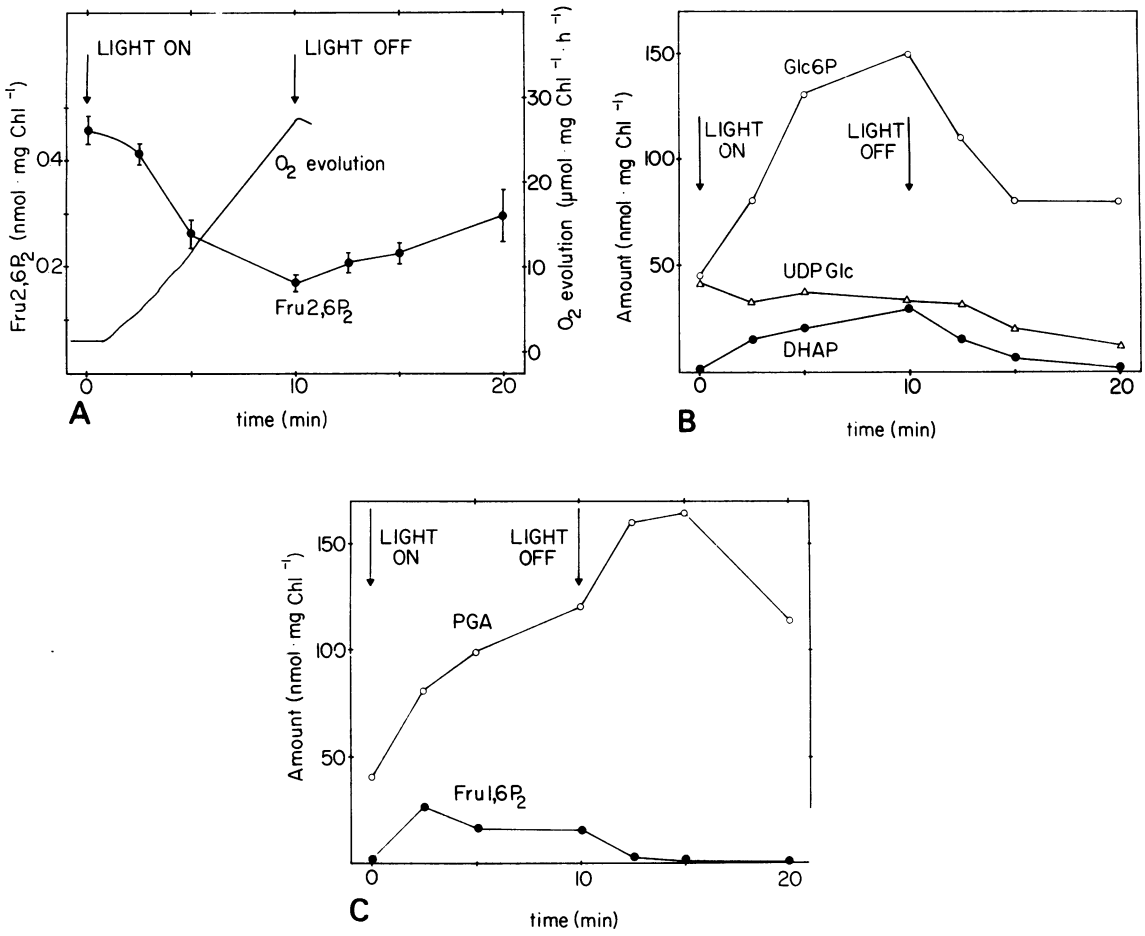

FIG. 1. Fru 2,6-P_2 and metabolite levels in spinach leaf discs during a dark-light-dark transition. Discs from leaves kept in the dark for 17 h were illuminated (190 w·m^{-2}) in saturating CO_2 for 10 min, and then darkened. A, Fru 2,6-P_2 and O_2 evolution; B, Glc 6-P, UDP glucose, and DHAP; C, Fru 1,6-P_2 and PGA. Results are mean of four separate samples.

Table I. *Fructose 2,6-bisphosphate Content of Leaf Discs after Lowering of the CO_2 Concentration*

Spinach leaf discs were cut from leaves predarkened for 17 h, and illuminated (190 w·m^{-2}) in a O_2 electrode for 20 min in saturating CO_2 or limiting CO_2. The CO_2 was provided by 300 μl of 1 M $NaHCO_3$ (pH 9) or by H_2O, respectively. Results are mean ± SE of four replicates.

	Photosynthesis	Fru 2,6-P_2
	μmol O_2·mg^{-1} Chl·h^{-1}	nmol·mg^{-1} Chl
20 min, saturating CO_2	177	102 ± 27
10 min saturating CO_2 and 10 min limiting CO_2	51	145 ± 12

for leaves. In recent detailed studies, chloroplasts have been isolated nonaqueously from spinach leaves carrying out photosynthesis in a range of different light intensities and CO_2 concentrations (Dietz and Heber, unpublished). It was shown here also, that high levels of stromal metabolites could be maintained when photosynthesis was low or inhibited. All these observations clearly show that sucrose synthesis is regulated in response to the rate of photosynthesis, and that one site for this regulation is the cytosolic FBPase. The 2- to 5-fold increase of Fru 2,6-P_2 in conditions when photosynthesis decreases could contribute to this regulation but the question arises as to why the Fru 2,6-P_2 is increasing.

Control of Fru 2,6-P_2 Concentration by C_3 Metabolites like DHAP. Studies on the isolated Fru 6-P, 2-kinase from spinach leaves, showed that DHAP was a very effective inhibitor (20). At concentrations of 0.25, 0.5, and 1 mM, respectively, DHAP inhibited Fru 6-P, 2-kinase by about 50, 70, and 82%, respectively. The measurements of DHAP and Fru 2,6-P_2 in spinach leaves provide evidence that the regulation of Fru 6-P, 2-kinase by DHAP has an important role *in vivo*. Figure 4 summarizes the relation between DHAP and Fru 2,6-P_2 in the experiments carried out using different light intensities or CO_2 concentrations (from Figs. 2 and 3). In these experiments, the DHAP content varied from less than 5 nmol/mg Chl in the dark up to 20 to 30 nmol/mg Chl during rapid photosynthesis, while the Fru 2,6-P_2 content decreased about 4-fold. Assuming a cytosolic volume of about 20 μl/mg Chl, these results suggest that the Fru 2,6-P_2 level *in vivo* indeed decreases markedly as the DHAP concentration varies between 0 and 1 mM. Some of the DHAP will be present in the stroma, but the alterations in the total amount of DHAP are so great that unless the vast majority of the increment in the light remains in the stroma, the conclusions drawn from the overall tissue contents will still be valid. In any case, this seems unlikely as DHAP is preferentially located in the cytosol (9, 15, 18) in the light.

PGA can also inhibit Fru 6-P, 2-kinase, and this inhibition is relieved by Pi (3, 20). Any increase in the cytosolic PGA during rapid photosynthesis would provide an additional mechanism for lowering the level of Fru 2,6-P_2, especially if the cytosolic Pi decreases simultaneously (18). In most of the conditions studied in our experiments, PGA is probably a less effective regulator of Fru 6-P, 2-kinase than DHAP. The concentration of PGA in the cytosol is lower than that of DHAP in the light (18), and the

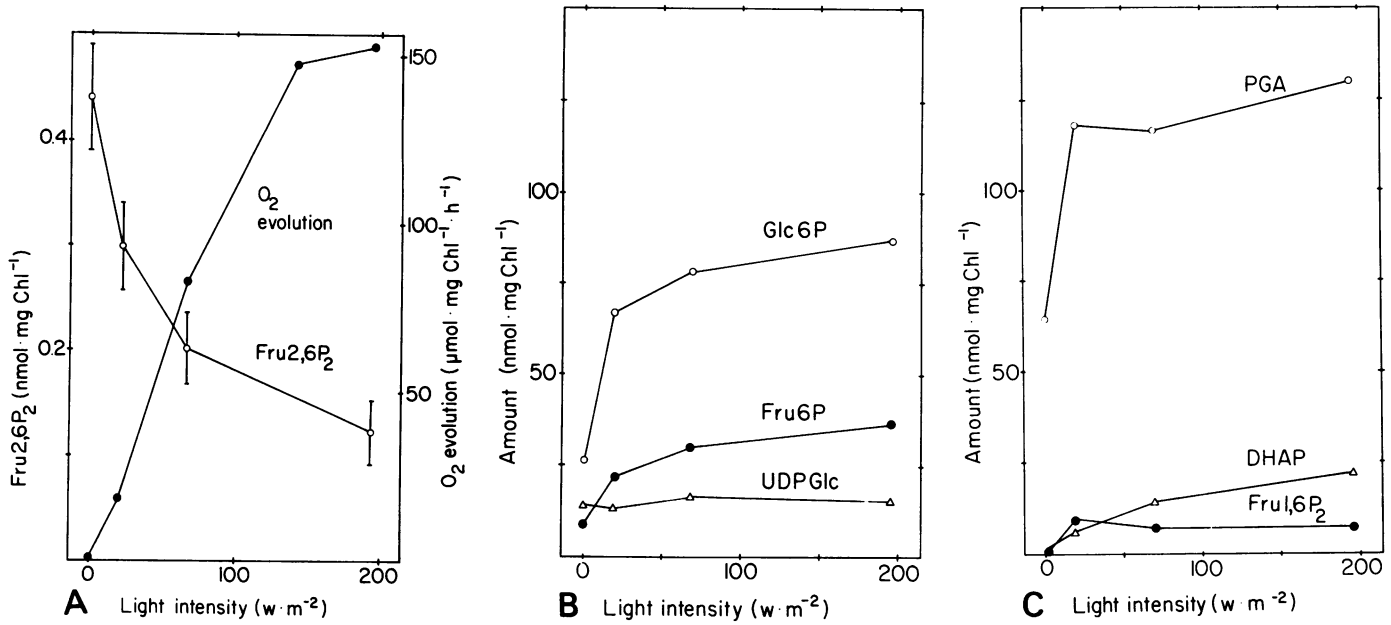

FIG. 2. Fru 2,6-P_2 and metabolite levels in spinach leaf discs carrying out photosynthesis at different light intensities. Leaf discs were illuminated at four different light intensities (see figure) in saturating CO_2 for 10 min. A, O_2 evolution and Fru 2,6-P_2. B, Glc 6-P, Fru 6-P, UDP glucose. C, PGA, DHAP, Fru 1,6-P_2. Results are mean of four separate samples.

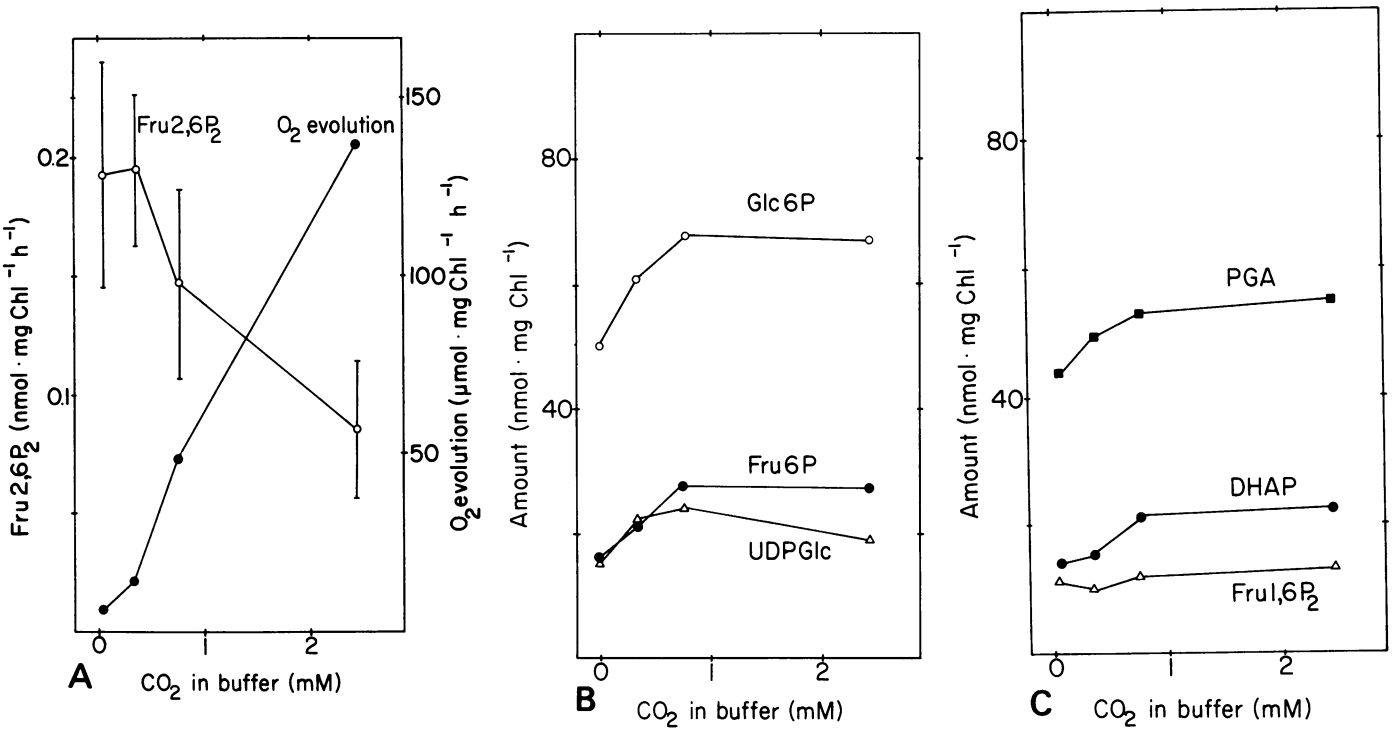

FIG. 3. Fru 2,6-P_2 and metabolite levels in spinach leaf discs carrying out photosynthesis at different carbon dioxide concentrations. Leaf discs were illuminated (190 w · m^{-2}) in varying CO_2 concentrations for 10 min. A, O_2 evolution and Fru 2,6-P_2; B, Glc 6-P, Fru 6-P, and UDP glucose; C, PGA, DHAP, Fru 1,6-P_2.

inhibitory effect of this PGA will be further diminished by Pi in the cytosol. The cytosolic concentration of PGA may sometimes even increase in the dark (18, Gerhardt unpublished). When photosynthesis is inhibited by limiting CO_2, however, a larger decrease in the PGA is found, both in the whole tissue (Fig. 3C) as well as in the cytosol of protoplasts (18). In these conditions, a lowered PGA concentration might make a larger contribution to the observed rise in Fru 2,6-P_2.

It should be noted that Fru 6-P is an activator of the Fru 6-P, 2-kinase; and also inhibits the Fru 2,6-bisphosphatase (3, 4). Since there is a decrease in hexose P when photosynthesis declines (Ref. 18; see also Figs. 1-3), this might be expected to favor a decrease in Fru 2,6-P_2. However, in these experiments with spinach leaf material carrying out photosynthesis in limiting conditions, the alterations of other metabolites like DHAP, PGA, and Pi more than compensate for the rise in Fru 6-P. In varying light, the alterations in DHAP are far larger than those of hexose P. In limiting CO_2, the alteration of DHAP is less marked, although still larger than those of the hexose P, and will be reinforced by a fall in the PGA/Pi quotient which should favor

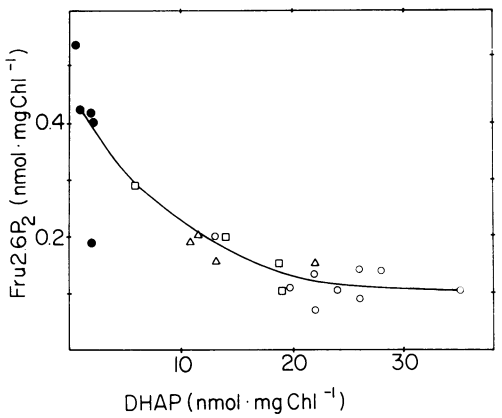

FIG. 4. Relation between Fru 2,6-P_2 and DHAP levels in spinach leaf discs. The results are from Figures 2 and 3 and other similar experiments and represent leaf discs in the dark (●), in limiting light (□), in limiting CO_2 (△), and in saturating conditions (○) after 10 min.

Fru 2,6-P_2 accumulation (see above).

In experiments which will be presented elsewhere (Cseke, Stitt, and Buchanan, in preparation), the activity of Fru 6-P, 2-kinase and Fru 2,6-bisphosphatase has been assayed in the presence of DHAP, PGA, Pi and Fru 6-P concentrations typical of those found in the dark, in the light with limiting CO_2, or in the light with saturating CO_2. The quotient of Fru 6-P, 2-kinase:Fru 2,6-bisphosphatase activity was lowest in the conditions simulating rapid photosynthesis and increased in conditions simulating those found when photosynthesis is limited by low light or CO_2, in agreement with the results presented here showing that the Fru 2,6-P_2 level decreases in rapid photosynthesis, and increases as light or CO_2 become limiting. This agreement between the *in vivo* levels and the properties of the partially purified enzymes which make and degrade Fru 2,6-P_2 confirms that alteration in the level of C_3 metabolites like DHAP and PGA is an important factor in controlling the Fru 2,6-P_2 concentration in response to the rate of photosynthesis, although the presence of further, as yet undiscovered, mechanisms can still not be excluded.

On the Contribution of Fru 2,6-P_2 to Regulation of Sucrose Synthesis during Photosynthesis in Limiting Conditions. These results point to a double role for Fru 2,6-P_2 in controlling the rate of sucrose synthesis in response to changes in the rate of photosynthesis. On the one hand, the presence of μM concentrations of Fru 2,6-P_2 in all the conditions studied means that the cytosolic FBPase will always be sensitive to inhibition by alterations in the concentrations of AMP or Fru 1,6-P_2 in the cytosol. When photosynthesis decreases, the concentration of DHAP decreases (Figs. 1–3; Ref. 18) and, since the reactions catalyzed by aldolase and triose P isomerase are close to equilibrium *in vivo*, the Fru 1,6-P_2 in the cytosol also decreases (15, 18). This can be confirmed by measurements of the cytosolic Fru 1,6-P_2 level (see Ref. 13 for details). It should be noted that the decrease of the cytosolic Fru 1,6-P_2 is masked in measurements of Fru 1,6-P_2 in whole leaf tissue as up to 90% of the Fru 1,6-P_2 is located in the stroma (9, 15, 18) and this does not alter or even increases when photosynthesis is inhibited (18; Dietz and Heber, unpublished). In addition, the concentration of Fru 2,6-P_2 itself often increases as the rate of photosynthesis declines, as decreasing concentrations of C_3 metabolites like DHAP and PGA will lead to a higher activity of Fru 6-P, 2-kinase, the enzyme which synthesizes Fru 2,6-P_2 (see above). This will provide an added sensitivity to the control of sucrose synthesis, as an alteration in the DHAP in the cytosol will generate reciprocal changes in the cytosolic concentratios of Fru 1,6-P_2 and Fru 2,6-P_2. The subsequent large alteration of the Fru 1,6-P_2:Fru 2,6-P_2 quotient could allow a sensitive modulation of cytosolic FBPase in response to alterations in the DHAP concentration.

Stimulation of the cytosolic FBPase would lead to increased production of hexose P. It has recently been shown that spinach leaf sucrose P synthase is stimulated by glucose 6-P (6) and inhibited by Pi (6, 11, 23). It appears that, by altering the activity of the FBPase, alterations in the concentration of glucose 6-P can be produced (Figs. 1B, 2B, and 2C) which in turn modulate the activity of sucrose P synthetase. In this way, a feed forward control of sucrose synthesis might be envisaged which operates to couple the rate of withdrawal of triose P from the chloroplast with the rate at which it is being generated in photosynthesis, and allows the pool size of Calvin cycle intermediates to be maintained at adequate levels over a wide range of conditions.

Two further aspects need to be considered, however, in assessing the significance of this feed forward control. First, all these experiments were carried out using leaf material which had been pretreated so that it contained low levels of sucrose, and Fru 2,6-P_2 was measured after short periods of photosynthesis so that any effects of accumulating sucrose could be minimized. A more complicated situation might arise when the leaf tissue contains more sucrose and is carrying out rapid starch synthesis. Second, the alterations of Fru 2,6-P_2 are not large, being often only 2-fold, and it may be asked whether such changes are enough to produce significant alterations in the activity of the cytosolic FBPase. These problems will be examined in the two following papers.

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