

A Role for Fructose 2,6-Bisphosphate in Regulating Carbohydrate Metabolism in Guard Cells¹

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ABSTRACT

Fructose 2,6-bisphosphate (Fru2,6P₂) appears to function as a regulator metabolite in glycolysis and gluconeogenesis in animal tissues, yeast, and the photosynthetic cells of leaves. We have investigated the role of Fru2,6P₂ in guard-cell protoplasts from *Vicia faba* L. and *Pisum sativum* L. (Argenteum mutant), and in epidermal strips purified by sonication from all cells except for the guard cells. Guard-cell protoplasts were separated into fractions enriched in cytosol and in chloroplasts by passing them through a nylon net, followed by silicone oil centrifugation. The cytosol contained a pyrophosphate: fructose 6-phosphate phosphotransferase (involved in glycolysis) which was strongly stimulated by Fru2,6P₂. A cytosolic fructose 1,6-bisphosphatase (a catalyst of gluconeogenesis) was inhibited by Fru2,6P₂. There was virtually no fructose 1,6-bisphosphatase activity in guard-cell chloroplasts of *V. faba*. It is therefore unlikely that the starch formed in these chloroplasts originates from imported triose phosphates or phosphoglycerate.

The level of Fru2,6P₂ in guard-cell protoplasts and epidermal strips was about 0.1 to 1 attomole per guard cell in the dark (corresponding to 0.05 to 0.5 nanomole per milligram chlorophyll) and increased three- to tenfold within 15 minutes in the light. Within the same time span, hexose phosphate levels in guard-cell protoplasts declined to approximately one-half, indicating that acceleration of glycolysis involved stimulation of reactions using hexose phosphates. The level of Fru2,6P₂ in guard cells appears to determine the direction in which carbohydrate metabolism proceeds.

Guard cells provide an attractive system for studying the control of glycolysis and gluconeogenesis in plants. Quite apart from the intrinsic interest in understanding how stomatal opening and closure can be regulated, they also provide one of the few cases in plants where large alterations in the rates of glycolysis and gluconeogenesis can be experimentally or naturally induced within a time scale of minutes. During stomatal opening (for reviews see 13, 16) malate is produced as a counterion to K⁺ by glycolytic breakdown of carbohydrates such as starch. During closure of the stoma the malate is released from guard cells or degraded. Some of it returns, via gluconeogenesis, to starch while another part may be respired to CO₂. No 3-phosphoglyceric acid or triose phosphates are produced by photosynthesis, because the reductive pentose phosphate path does not operate in guard cells (14, 17); this makes stomata a system where a rapid and controllable alteration between glycolysis and gluconeogenesis can be produced in response to the various treatments which affect stomatal aperture, including illumination, CO₂, and ABA.

Although other reactions are certainly involved, a key step in controlling the activity of glycolysis and gluconeogenesis are the reactions which interconvert hexose phosphate and triose phosphate, namely those catalyzed by PFK² (in the glycolytic direction) and Fru1,6P₂ase (in the gluconeogenetic direction). Although the activity of these enzymes can be modulated by adenine nucleotides and phosphorylated intermediates (15, 25), work on liver (6, 7) and subsequently on other tissues, including plant leaves (2), has shown that a recently discovered regulator metabolite called Fru2,6P₂ plays a crucial role in controlling these activities. Generally, an increase of Fru2,6P₂ tends to promote glycolysis at the expense of gluconeogenesis. Fru2,6P₂ is a potent inhibitor of Fru1,6P₂ase in animals (7) as well as plants (3, 10, 24), and an activator of the PFK in animals (6). For plants, it seems that the ATP-dependent PFK is not stimulated by Fru2,6P₂, however a PPI dependent phosphorylation of Fru6P catalyzed by PFP is strongly activated by Fru2,6P₂ (3, 9, 24).

The aim of the work described in this article was to establish whether Fru2,6P₂ could be involved in controlling carbohydrate metabolism during the opening and closing movements of stomata. We have used purified preparations of isolated guard-cell protoplasts prepared from leaves of *Vicia faba* and the Argenteum mutant of *Pisum sativum* as well as sonicated epidermal strips from *V. faba* to determine how much Fru2,6P₂ is present in these cells, and to study the subcellular location, activity, and sensitivity to Fru2,6P₂ of PFP and the Fru1,6P₂ase.

MATERIALS AND METHODS

Plants. Seeds of *Vicia faba* L. (cv Weißkeimige Hangdown) and *Pisum sativum* L. (Argenteum mutant, 12) were germinated and grown in a potting mixture in the greenhouse at 20°C (day) and 17°C (night). When necessary, the natural photoperiod was extended to 14 h by metal halide lamps (Osram HQI-TS 250 W/D).

Preparation of Epidermal Strips and Guard Cell Protoplasts. Protoplasts from *V. faba* were prepared by a modification of the method of Shimazaki *et al.* (19). The lower epidermis of fully expanded bifoliolate leaves from plants 2 to 3 weeks old was peeled off and sonicated for 30 to 60 s with an ultrasonic disrupter (Branson B 15) to rupture epidermal cells and to remove the mesophyll that adhered to the epidermal strips. The cell walls were digested with 2% Rohament TC (cellulase), 0.5% Rohament P (pectinase), 0.5% BSA, 0.4 M mannitol, 10 mM Na ascorbate,

² Abbreviations: PFK, phosphofruktokinase; Fru6P, fructose 6-phosphate; Fru1,6P₂, fructose 1,6-bisphosphate; Fru1,6P₂ase, fructose 1,6-bisphosphatase; Fru2,6P₂, fructose 2,6-bisphosphate; PFP, pyrophosphate:fructose 6-phosphate phosphotransferase; Rubisco, ribulose 1,5-bisphosphate carboxylase-oxygenase.

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and 1 mM CaCl₂ at 25°C with gentle agitation. After 60 to 90 min the guard cell protoplasts were released from the epidermal strips into the incubation medium. To separate the guard cell protoplasts (15–20 μm diameter) from the strips the incubation solution was passed through a 20 μm nylon net. The filtrate (containing guard cell protoplasts, chloroplasts, and cell debris) was centrifuged at 80g for 5 min. The pellet was resuspended in 0.5 M mannitol, recentrifuged at 50g for 5 min, and the sediment (containing only guard cell protoplasts) was suspended in the fractionation or assay media.

Guard cell protoplasts of *P. sativum* were prepared by a modification of the method of Jewer *et al.* (8). After peeling the lower epidermis of 4- to 5-week old plants, the epidermal strips were incubated for 30 min at 25°C with gentle agitation in a solution containing 1% Rohament TC, 0.5% Rohament P, 0.5% BSA, 0.35 M mannitol, 10 mM Na ascorbate, and 1 mM CaCl₂. To remove protoplasts of the common epidermal cells, the medium was poured onto a 200 μm net. The remaining epidermal peels (still containing the guard cells) were washed twice with 0.3 M mannitol and incubated for another 30 min in the isolation mixture given above. Then the incubation medium was passed through a 14 μm net to separate the guard cell protoplasts (in the filtrate, 10–15 μm diameter) from the larger epidermal-cell protoplasts (30–60 μm diameter) and epidermal peels (on the net). The filtrate was spun at 100g for 5 min, the pellet dispersed in 0.3 M mannitol, and centrifuged at 50g for 5 min. Cell debris and chloroplasts were removed by decanting the supernatant. The sediment (containing the guard cell protoplasts) was resuspended in the fractionation or assay media.

Cell numbers were counted in a hemocytometer. Contamination by mesophyll protoplasts was determined by measuring Rubisco activity and relating it to the amount of Chl in the sample. Relative Rubisco activity of guard cell preparations was 5% of that of mesophyll protoplasts. The Chl content of a guard cell protoplast was about 1 pg in *V. faba* and 0.75 pg in *P. sativum*.

Fractionation of Guard Cell Protoplasts. To separate the chloroplasts from the cytosol the guard cell protoplasts from *V. faba* and *P. sativum* were forced through a 5 μm nylon net. The filtrate was rapidly spun through a silicone oil layer (Wacker Chemie AR 147) for 30 s, or at 3500 rpm for 3 min without silicone oil. After any one of the two centrifugations the supernatant was enriched in cytosolic and the pellet in chloroplastic enzymes.

Determination of Enzyme Activities and of Fru2,6P₂ Levels. Activities of phosphoenolpyruvate carboxylase, malic enzyme, and NADPH-linked malate dehydrogenase were measured as described by Willmer *et al.* (28); activity of NADPH glyceraldehyde phosphate dehydrogenase was assayed according to Wirtz *et al.* (29), and activities of ATP-PPK, PFP, and Fru1,6P₂ase according to Stitt *et al.* (20, 24). Modifications will be mentioned in the "Results" section. For quantitative determinations of hexose phosphates, triose phosphates, and 3-phosphoglycerate, protoplast suspensions were treated with HClO₄, final concentration 10% (v/v), then neutralized with 5 N KOH and 1 N triethanolamine, centrifuged, and assayed by coupled enzymic reactions (29).

Rubisco activity was determined as incorporation of ¹⁴C from [¹⁴C]HCO₃ into acid-stable products, following the procedure of Lorimer *et al.* (11).

Fru2,6P₂ was extracted and assayed using the method of Van Schaftingen *et al.* (27) as adapted to maize leaves (22). Briefly, samples were quenched in a cooled chloroform-methanol mixture. After addition of alkaline buffer, Fru2,6P₂ partitioned into the aqueous phase. Amounts of Fru2,6P₂ were determined by the activation of PFP prepared from potato tubers (27). The assay was calibrated with known amounts of Fru2,6P₂. Small

amounts of Fru2,6P₂ added to the suspension of the guard cell protoplasts at the time of injection of the killing mixture were recognized with an efficiency ranging between 80 and 100% of the additions.

Chemicals. Rohament TC and Rohament P were obtained from Röhm GmbH (Darmstadt, W. Germany), silicone oil from Wacker Chemie (Munich, W. Germany), nylon net from Heidländ (Guetersloh, W. Germany), and the enzymes for coupled enzyme assays from Boehringer (Mannheim, W. Germany). All other chemicals were from Sigma (Munich, W. Germany).

RESULTS

Presence of Fru2,6P₂ in Guard Cells. Preliminary experiments showed appreciable levels of Fru2,6P₂ in sonicated epidermal strips of *V. faba* (of the order of 1–2 nmol/mg Chl) and in guard cell protoplasts from *V. faba* and *P. sativum* (0.05–5 nmol/mg Chl, and the data of Table I). The amounts varied greatly, depending especially on illumination; we will elaborate on this further below. The Fru2,6P₂ discovered in epidermal strips and guard cell protoplasts was not introduced into the samples as contamination from the mesophyll tissue. In epidermal strips and in guard cell protoplasts, Rubisco activity, per unit Chl, was only 0.05 times as high as in whole leaves. In guard cell protoplasts of *V. faba*, the amounts of Fru2,6P₂ were 2 to 12 times higher than in mesophyll tissue of the same species. Clearly, Fru2,6P₂ was present in guard cells.

Table I. Fru2,6P₂ Contents of Guard Cell Protoplasts (GCP) from *V. faba* and *P. sativum* in the Light and in Darkness

Protoplasts were illuminated or darkened for 15 min at 20°C before quenching metabolism by addition of a cold chloroform-methanol mixture. Light from a slide projector that saturated photosynthesis in mesophyll protoplasts (quantum flux 1 mmol m⁻² s⁻¹). Means ± SE, from 8 (*V. faba*) or 6 (*P. sativum*) separate preparations of protoplasts.

	Fru2,6P ₂ (amol/GCP)		Light Dark
	Light	Dark	
<i>V. faba</i>	2.42 ± 0.38	0.39 ± 0.14	6.2
<i>P. sativum</i>	0.58 ± 0.09	0.08 ± 0.03	6.9

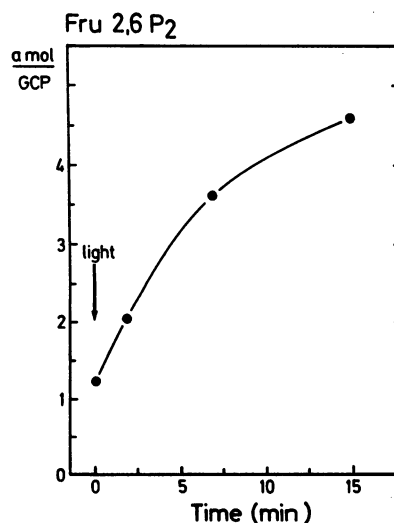


FIG. 1. Time course of the light-dependent increase of Fru2,6P₂ in guard cell protoplasts from *V. faba*. Protoplasts were incubated at 20°C in the dark for 15 min before the light was turned on (illumination by a slide projector, quantum flux 1 mmol m⁻² s⁻¹). Samples were withdrawn at the indicated times and then quenched in a cold chloroform-methanol mixture. One experiment out of 3.

Upon illumination, Fru2,6P₂ contents rose in guard cell protoplasts from both *V. faba* and from *P. sativum* (Table I). In both species, the increase in the light varied between 3- and 10-fold among the individual experiments. After the light was turned off, levels of Fru2,6P₂ declined to the previous level in the dark. Alterations in the Fru2,6P₂ content between dark and light occurred rapidly, so that 15 min in the light or in darkness sufficed as standard treatments. Figure 1 gives an example for a 4-fold increase of Fru2,6P₂ content within this time span. In general, Fru2,6P₂ contents were lower in guard cells of *P. sativum* than in those of *V. faba*; similar differences appeared also between the levels of other metabolites and between enzyme activities, as we will show below.

Intracellular Distribution of Enzymes. We next studied the intracellular localization of the enzymes which catalyze the conversion of Fru6P to Fru1,6P₂, and the reverse reactions from Fru1,6P₂ to Fru6P, and whose activity could be regulated by Fru2,6P₂. To do this, fractions enriched in chloroplastic or extrachloroplastic material were prepared by passing guard cell protoplasts through a 5 μm net to disrupt the plasmalemma and then separating the intact chloroplasts from the remaining cellular debris by centrifugation through silicone oil. The cross-contamination between the chloroplast fraction (pellet) and extrachloroplastic fraction (supernatant) was determined by measuring the distribution of marker enzymes for the cytosol (phosphoenolpyruvate carboxylase and malic enzyme) and the chloroplasts (NADP-malate dehydrogenase and NADP-glyceraldehyde 3-phosphate dehydrogenase). Contamination of the chloroplast pellet by cytosolic enzymes was negligible. However, the supernatant (cytosol) showed an activity of chloroplastic enzymes as large as 30% of that found in the chloroplast fraction. This contamination is higher than the one determined in leaf mesophyll protoplasts; it was probably caused by the relation in size between guard-cell protoplasts (diameter 15–20 μm) and chloroplasts (2–4 μm). A 5 μm net, which is small enough to break guard cell protoplasts, will also break some chloroplasts.

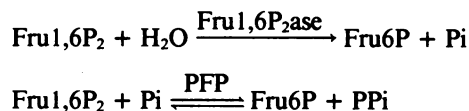
The compilation in Table II indicates that the ATP-PFK was present in the sediment which consisted mainly of the chloroplasts, but the majority of the ATP-PFK activity occurred outside of these organelles. This distribution of ATP-PFK activity is similar to that reported for green leaves (25) and endosperm tissue (10). Activity of PFP, as measured in the glycolytic direction, occurred predominantly in the cytosol, as was reported for leaves (4), and it should be noted that the activity of the PFP was well in excess of that of the ATP-PFK.

Hydrolysis of Fru1,6P₂ to Fru6P was catalyzed only by the cytosolic fraction; the chloroplast fraction was inactive in this respect. The possibility existed that the assay conditions chosen by us were unfavorable for the discovery of a Fru1,6P₂ase in the

chloroplasts. We conducted additional experiments in which we attempted to simulate chloroplastic conditions (1). The pH was raised from 7.0 to 8.5. The concentration of Mg²⁺ was increased to 10 mM, that of Fru1,6P₂ to 3 mM, and 10 mM DTT was included. Hydrolysis of Fru1,6P₂ remained negligible, neither did darkening or illuminating the protoplasts for 15 min prior to fractionation lead to the appearance of a Fru1,6P₂ase activity. The capacity of guard cells to hydrolyze Fru1,6P₂ appears to reside in their cytosol. The properties of the enzymes responsible for this capacity were then studied in more detail. Because the activity responsible for hydrolysis of Fru1,6P₂ was virtually absent from the chloroplasts, the investigation of Fru2,6P₂-sensitive enzymes was continued on guard cell protoplasts without further fractionation. We began with PFP, which is one of the two enzymes known to be modulated in their activities by Fru2,6P₂, and followed up with experiments on the cytosolic Fru1,6Pase, the other Fru2,6P₂-regulated enzyme.

Stimulation of PFP by Fru2,6P₂. The activity of PFP in the glycolytic direction depended almost totally on Fru2,6P₂ (Fig. 2). With 1 μM Fru2,6P₂ present, the pH optimum was at about 8 and the apparent *K_m* for Fru6P was about 0.7 mM.

The hydrolysis of Fru1,6P₂ catalyzed by Fru1,6P₂ase is irreversible. In contrast, PFP catalyzes a reversible reaction, so that it could also catalyze a Pi-dependent hydrolysis of Fru1,6P₂:



To study this backreaction of PFP, we searched for assay conditions which allowed a separation of the contributions made by PFP and by the cytosolic Fru1,6P₂ase. Because of the high affinity of the PFP for Pi and the unavoidable contamination of the assay mixtures with Pi, activity of PFP could not be suppressed by excluding Pi. Activity of PFP was distinguished from a Fru1,6P₂ase activity by its differing dependences on (a) Fru1,6P₂, (b) Fru2,6P₂, (c) AMP and Cl⁻, and (d) pH: (a) In the absence of Fru2,6P₂, Fru1,6P₂ase has a higher affinity for Fru1,6P₂ than PFP (Fig. 3). (b) Presence of Fru2,6P₂ inhibits Fru1,6P₂ase and stimulates the backreaction of PFP (Fig. 4). (c) Fru1,6P₂ase is inhibited by AMP but not by Cl⁻. The backreaction of PFP is insensitive to AMP but inhibited by many anions including Cl⁻ (27, and data not shown), (d) The cytosolic Fru1,6P₂ase is less active at neutral or slightly acidic pH values especially at low Mg²⁺; in contrast, PFP remains active under these conditions (Fig. 3).

When guard cell protoplasts were assayed for their ability to catalyze the hydrolysis of Fru1,6P₂ in the absence of Fru2,6P₂, two separate relationships to the pH of the assay medium

Table II. *Distribution and Activity of Enzymes in Guard Cell Protoplasts of V. faba*

Guard cell protoplasts (GCP) were forced through a 5 μm nylon net and then centrifuged through a silicone oil layer. Hydrolysis of Fru1,6P₂ was measured at pH 8 in the presence of 5 mM MgCl₂ and therefore represents primarily the activity of Fru1,6P₂ase. One out of four experiments conducted with similar results.

Enzyme	Relative Activity in Fraction % of Total Activity		Activity in Compartment pmol per GCP and h	
	Sediment	Supernatant	Sediment	Supernatant
Phosphoenolpyruvate carboxylase	1	99	0.1	9
NADP-malic enzyme	0.2	99.8	0.001	0.6
NADP-malate dehydrogenase	72	28	0.42	0.16
NADP-glyceraldehyde 3-phosphate dehydrogenase	65	35	2.8	0.15
ATP-PFK	27	73	1.7	4.6
PFP	2	98	0.2	10
Fru1,6P ₂ -hydrolysis	0.5	99.5	0.003	0.6

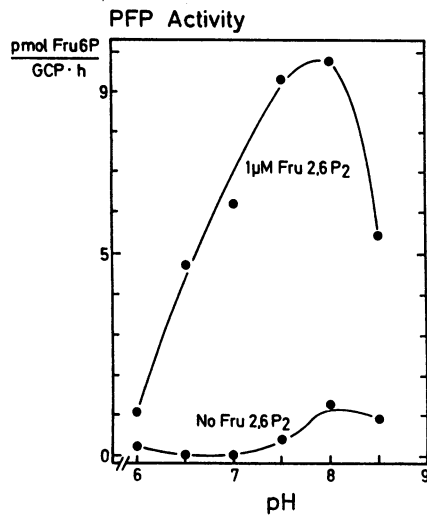


FIG. 2. Dependence of PFP activity on pH in the presence and absence of $1 \mu\text{M}$ Fru $_{2,6}\text{P}_2$. Guard cell protoplast of *V. faba*.

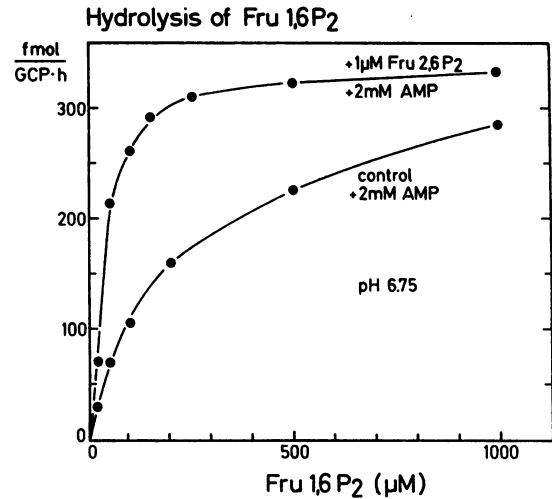


FIG. 4. Effect of $1 \mu\text{M}$ Fru $_{2,6}\text{P}_2$ on the concentration dependence of Fru $_{1,6}\text{P}_2$ hydrolysis at pH 6.75 in the presence of 2 mM AMP and 5 mM MgCl_2 . Guard cell protoplasts of *V. faba*.

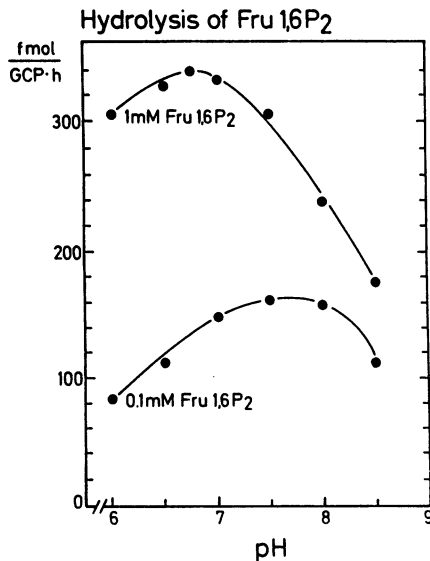


FIG. 3. Dependence of the hydrolysis of Fru $_{1,6}\text{P}_2$ on pH in the presence of 1 mM and 0.1 mM Fru $_{1,6}\text{P}_2$. Activity was measured in the presence of 5 mM MgCl_2 . Guard cell protoplasts of *V. faba*.

emerged, depending on the level of added Fru $_{1,6}\text{P}_2$ (Fig. 3). When the Fru $_{1,6}\text{P}_2$ concentration was high (1 mM), the optimal pH for hydrolysis was 6.75. At a low Fru $_{1,6}\text{P}_2$ concentration (0.1 mM), the optimal pH was 8. In further experiments, conducted at pH 6.75, we found that hydrolysis was stimulated by Fru $_{2,6}\text{P}_2$, which lowered the apparent K_m for Fru $_{1,6}\text{P}_2$ from about 200 to about $40 \mu\text{M}$ (Fig. 4). This behavior resembles that of PFP in spinach leaves (3), potato tubers (7), and castor bean cotyledons (9), in all of which Fru $_{2,6}\text{P}_2$ activated the back reaction by lowering the K_m for Fru $_{1,6}\text{P}_2$. The hydrolysis of 1 mM Fru $_{1,6}\text{P}_2$ was not inhibited by 2 mM AMP at pH 6.75 but addition of 40 mM NaCl reduced the hydrolytic activity to $<20\%$ of the control value (data not shown). These results provide evidence for the presence of a back reaction of PFP which can convert Fru $_{1,6}\text{P}_2$ to Fru6P in guard cells. We conclude that stimulation of the catalytic activity of PFP by Fru $_{2,6}\text{P}_2$ could affect the formation of Fru $_{1,6}\text{P}_2$ as well as its hydrolysis.

Inhibition of the Cytosolic Fru $_{1,6}\text{P}_2$ ase by Fru $_{2,6}\text{P}_2$. The presence of a classical cytosolic Fru $_{1,6}\text{P}_2$ ase which can be inhibited by Fru $_{2,6}\text{P}_2$ and AMP was investigated at pH 8. A pH of 8

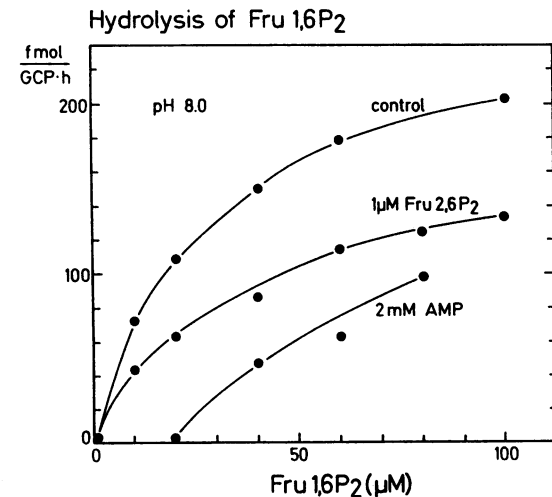


FIG. 5. Inhibition by AMP and Fru $_{2,6}\text{P}_2$ of Fru $_{1,6}\text{P}_2$ hydrolysis at pH 8.0. Guard cell protoplasts of *V. faba*.

and low concentrations of Fru $_{1,6}\text{P}_2$ should be optimal for testing this enzyme because the activity of PFP is then partially suppressed (Fig. 3). Under these conditions, $1 \mu\text{M}$ Fru $_{2,6}\text{P}_2$ or 2 mM AMP strongly inhibited the hydrolysis of Fru $_{1,6}\text{P}_2$ (Fig. 5). The addition of 40 mM NaCl did not cause a further inactivation. The apparent K_m for Fru $_{1,6}\text{P}_2$ was about $20 \mu\text{M}$. This K_m value is five times higher than that obtained for the cytosolic Fru $_{1,6}\text{P}_2$ ase from leaves of spinach (23) or wheat (24), but equals that of the Fru $_{1,6}\text{P}_2$ ase from maize mesophyll cells (22) and castor bean endosperm (10).

Activation of Glycolysis by Illumination. Important regulatory enzymes can be identified *in vivo* by measuring the concentrations of metabolites when fluxes through a pathway are changing. If stimulation of a pathway is accompanied by a decrease in the levels of selected intermediates, we may conclude that there has been an activation of the enzymes for which these intermediates act as substrates. Thus, an activation of glycolysis by stimulation of PFK or PFP should result in a depletion of the hexose-phosphate pool while the levels of triose phosphates should not be necessarily affected, as they would fluctuate, depending on the rates of triose-phosphate consumption. This pattern was indeed observed in guard cell protoplasts. Upon illumination, hexose-phosphate levels declined (Fig. 6) with about the same

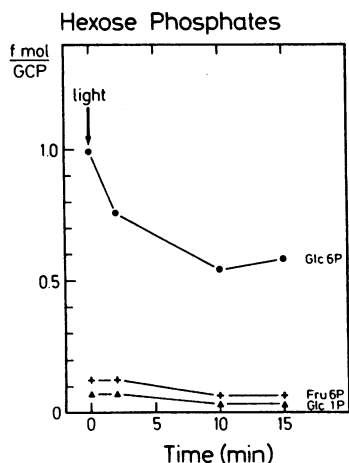


FIG. 6. Decline of hexose-phosphate levels in guard-cell protoplasts. Protoplasts from *V. faba* were incubated in darkness for 15 min, then illuminated (as in Fig. 1). At the indicated times, samples were drawn, their metabolism was stopped, and contents were assayed.

time constant as was typical for the increase of Fru2,6P₂ (Fig. 1). This decline of hexose phosphates was repeatedly observed. In contrast, the measured levels of Fru1,6P₂, triose phosphates, and 3-phosphoglycerate varied in an unpredictable manner or did not change at all (not shown).

DISCUSSION

Alterations of Fructose 2,6-Bisphosphate Contents in Guard Cells. Fru2,6P₂ levels in guard cells rose rapidly after illumination (Fig. 1) and declined after darkening. It is intriguing that illumination led to an increase in the Fru2,6P₂ content of guard cells to promote opening, in contrast to photosynthetic tissue where the Fru2,6P₂ content declines in the light in order to stimulate sucrose synthesis (23). The mechanisms controlling the levels of Fru2,6P₂ appear to differ in guard cells and in cells able to reduce CO₂.

The decline of Fru2,6P₂ following illumination has been attributed to higher levels of 3-phosphoglycerate and triose phosphates during photosynthesis (23) which inhibit the synthesis of Fru2,6P₂ by Fru6P,2-kinase. It will be shown elsewhere that the increase of Fru2,6P₂ in guard cell protoplasts in the light is probably not caused by alterations of such effector metabolites. Instead, an as yet unidentified mechanism leads to an alteration in the activation of Fru6P,2-kinase and Fru2,6P₂ phosphatase which can be detected if guard cell protoplasts are lysed and the extract assayed immediately (M. Stitt and K. Raschke, unpublished data).

We have not yet investigated the effects of factors other than light which affect stomatal opening but it is obvious that increased levels of Fru2,6P₂ could operate as a switch from gluconeogenesis to glycolysis inhibiting cytosolic Fru1,6P₂ase and stimulating PFP. The changes in Fru2,6P₂ levels we determined to occur between darkness and light were 3- to 10-fold, which is more than is required for the regulation of photosynthetic sucrose synthesis in leaves (21, 23). However, before considering how Fru2,6P₂ may be involved in stomatal movement we have to determine the location and activity of the key enzymes of glycolysis and gluconeogenesis in these cells, as all the evidence available at present, admittedly from photosynthesizing cells, indicates that Fru2,6P₂ will be restricted to the cytosol (4, 21).

Activity and Compartmentation of Glycolytic and Gluconeogenic Enzymes. The results of our experiments indicate that guard cells possess a strong glycolytic capacity which by far exceeds their capacity for gluconeogenesis. The combined activ-

ities of the ATP-PFK in the chloroplasts and the cytosol were larger than the activity of Fru1,6P₂ase by a factor of 10 (Table II). The apparent imbalance in favor of glycolysis increases if the high activity of the PFP is included in the assessment; the PFP capacity in the glycolytic direction even exceeded that of the ATP-PFK. Admittedly, PFP may also support gluconeogenesis by catalyzing the hydrolysis of Fru1,6P₂ in the back reaction, but the activity of PFP in the gluconeogenic direction is much smaller than in the glycolytic direction, as shown by the data in Table II and Figures 2 and 4. If the relative activities of the enzymes ATP-PFK, PFP, and Fru1,6P₂ase are suitable to assess catabolic and anabolic capacities we can interpret our results as indicating that guard cells of *V. faba* are amply equipped to rapidly break down carbohydrates for the production of malate as osmoticum for stomatal opening and counter-ion to K⁺ but that the capacity for the return of the carbon skeletons into carbohydrates during stomatal closure does not appear to be so high. We can compare rates of malate formation in guard cell protoplasts of *V. faba* with the activities of the enzymes involved in glycolysis and find that malate was synthesized at a rate of 0.2 pmol per guard cell and h (18). The activities of the ATP-PFK and of PFP we found (Table II) are much in excess of the required activities; the glycolytic capacity of guard cells appears to suffice for the observed rate of malate formation. If we turn to the measured Fru1,6P₂ase activity as an indicator of the gluconeogenic capacity (approximately 1 pmol per guard cell and h, Table II) and compare this with the value for the rate of malate breakdown during stomatal closing of 0.8 pmol per guard cell and h (derived from the data of Van Kirk and Raschke, 26) we recognize that the maximal activities of the cytosolic Fru1,6P₂ase and PFP could cope with the necessary fluxes, but they do lie considerably under the glycolytic capacity. Their activities *in situ* are also likely to be well below those measured *in vitro* under optimized assay conditions. Of course, oxidation of malate in the tricarboxylic acid cycle and release from the guard cells are other mechanisms of malate disposal available to the guard cells (5, 26).

Additional unexpected questions are raised by the localization of the glycolytic and gluconeogenic enzymes. The absence of a chloroplastic Fru1,6P₂ase activity from the guard cells poses a serious problem as to the form in which reduced carbon is transferred to guard-cell chloroplasts. It has also recently been observed that the majority of the enzymes responsible for degradation of starch are located outside the plastids of guard-cell protoplasts (J. Preiss, personal communication). While conclusive evidence is still lacking, it is possible that a major path to and from starch in guard cells involves the transport of compounds produced by the cytosolic Fru1,6P₂ase or acting as substrates for PFK, respectively. If this was so, it is easy to understand why the majority of the glycolytic path is found in the cytosol. It would also increase the potential significance of Fru2,6P₂ as a regulator of stomatal carbohydrate metabolism.

Possible Role of Fru2,6P₂ in Guard Cells. The striking rise in the Fru2,6P₂ level of guard cells in response to illumination could lead to an enhancement of glycolysis in the cytosol through an activation of the PFP (Fig. 2), and a suppression of gluconeogenesis through an inhibition of the cytoplasmic Fru1,6P₂ase (Fig. 5). Decline of Fru2,6P₂ in the dark would allow the Fru1,6P₂ase to become active in assisting the removal of malate during stomatal closure.

The rapid decline of the hexose phosphate level in guard cells (Fig. 6), which parallels the rise of Fru2,6P₂ after illumination (Fig. 1), provides evidence that enzymes have been activated that convert hexose phosphates to triose phosphates. We should not ignore complexities which arise as a result of intracellular compartmentation but the observation that Fru2,6P₂ rises so dramatically as hexose phosphate are being consumed make it very

tempting to suggest that at least part of this stimulation is due to the action of Fru2,6P₂ on cytosolic enzymes. Whereas it is clear that PFP activity depends on the presence of Fru2,6P₂, the cytosolic PFK is apparently not activated by Fru2,6P₂. We do not know whether a mechanism exists which would stimulate PFK. While the way in which Fru2,6P₂ modulates enzyme activity requires further attention, the results obtained on guard cells provide clear evidence that an increase of Fru2,6P₂ accompanies stimulation of carbohydrate mobilization and glycolysis, and that Fru2,6P₂ is implicated in activating these processes.

In conclusion, we suggest that Fru2,6P₂ has the role of a regulator metabolite in guard cells. It is involved in determining the direction in which carbohydrate metabolism proceeds when guard cells inflate or shrink. A new question arises: how are production and metabolism of Fru2,6P₂ being controlled when stomata function as moderators of gas exchange? From a different point of view, guard cells should lend themselves to a clarification of the role of Fru2,6P₂ in the control of glycolysis in plants.

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