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Synthesis and Degradation of Fructose 2,6-Bisphosphate in Endosperm of Castor Bean Seedlings[1]

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NICHOLAS J. KRUGER[2] AND HARRY BEEVERS*
Biology Department, University of California, Santa Cruz, California 95064

ABSTRACT

The aim of this work was to examine the possibility that fructose 2,6-bisphosphate (Fru-2,6-P_2) plays a role in the regulation of gluconeogenesis from fat. Fru-2,6-P_2 is known to inhibit cytoplasmic fructose 1,6-bisphosphatase and stimulate pyrophosphate:fructose 6-phosphate phosphotransferase from the endosperm of seedlings of castor bean (*Ricinus communis*). Fru-2,6-P_2 was present throughout the seven-day period in amounts from 30 to 200 picomoles per endosperm. Inhibition of gluconeogenesis by anoxia or treatment with 3-mercaptopicolinic acid doubled the amount of Fru-2,6-P_2 in detached endosperm. The maximum activities of fructose 6-phosphate,2-kinase and fructose 2,6-bisphosphatase (enzymes that synthesize and degrade Fru-2,6-P_2, respectively) were sufficient to account for the highest observed rates of Fru-2,6-P_2 metabolism. Fructose 6-phosphate,2-kinase exhibited sigmoid kinetics with respect to fructose 6-phosphate. These kinetics became hyperbolic in the presence of inorganic phosphate, which also relieved a strong inhibition of the enzyme by 3-phosphoglycerate. Fructose 2,6-bisphosphatase was inhibited by both phosphate and fructose 6-phosphate, the products of the reaction. The properties of the two enzymes suggest that *in vivo* the amounts of fructose-6-phosphate, 3-phosphoglycerate, and phosphate could each contribute to the control of Fru-2,6-P_2 level. Variation in the level of Fru-2,6-P_2 in response to changes in the levels of these metabolites is considered to be important in regulating flux between fructose 1,6-bisphosphate and fructose 6-phosphate during germination.

During the early growth of castor bean seedlings there is a massive conversion of fat to sugar in the endosperm. The final stage of this conversion, the synthesis of sucrose from oxaloacetate, probably occurs exclusively in the cytoplasm (15, 16). Present evidence suggests that the production of Fru-6-P[3] from Fru-1,6-P_2 is an important regulated step in this sequence. This reaction is far removed from equilibrium *in vivo* (6) and the changes in the levels of intermediates in endosperm during anoxia (when gluconeogenesis is restricted and glycolysis is stimulated) indicate that the step is not limited solely by substrate concentration (6).

We recently studied the properties of the enzymes involved in the interconversion of Fru-1,6-P_2 and Fru-6-P during gluconeogenesis. Cytoplasmic FBPase is markedly inhibited by Fru-2,6-P_2, which also increases the sensitivity of the enzyme to inhibition by AMP and Pi (9). In contrast, PFP, which is confined to the cytoplasm (10), is enormously stimulated by the same effector (8). This latter enzyme may catalyze the reverse reaction (from Fru-6-P to Fru-1,6-P_2) *in vivo*. Thus, through its effects on PFP and FBPase, variation in the level of Fru-2,6-P_2 could regulate the flux from Fru-1,6-P_2 to Fru-6-P in the endosperm.

Fru-2,6-P_2 is important in the control of carbohydrate metabolism in both yeast and mammalian cells (5). In addition, present evidence suggests that it may contribute to the regulation of sucrose synthesis in spinach leaves, where cytoplasmic FBPase and PFP respond to Fru-2,6-P_2 in the same way as shown later for castor bean endosperm (4, 21). Moreover, the level of Fru-2,6-P_2 in spinach leaves varies in response to both illumination and the level of sugar in the tissue (20). Such changes in the amount of Fru-2,6-P_2 are probably regulated by control of the activities of Fru-2,6-P,2K and Fru-2,6-P_2ase (specific enzymes for the synthesis and degradation of Fru-2,6-P_2, respectively) which are present in spinach leaves (2, 3).

Less is known about the levels and metabolism of Fru-2,6-P_2 in nonphotosynthetic tissues. Fru-2,6-P_2 is present in etiolated mung bean hypocotyl (17), and its level in Jerusalem artichoke tubers increase in response to wounding (22). Fru-6-P,2K and Fru-2,6-P_2ase have been measured in Jerusalem artichoke tubers and in maize roots, respectively (18, 22). Here we report that castor bean endosperm tissue contains enzyme activities required for both the synthesis and degradation of Fru-2,6-P_2, and that changes in the level of Fru-2,6-P_2 in this tissue are consistent with its proposed role in the regulation of gluconeogenesis.

MATERIALS AND METHODS

Materials. Castor bean seeds (*Ricinus communis* L. cv Hale) were soaked for 24 h in running tap water, then placed in moist vermiculite and grown in the dark at 30°C in a humidified growth chamber. The plants were harvested 4 d after sowing, unless otherwise stated. Potato tubers (*Solanum tuberosum* L. cv Russet) and mung bean sprouts (*Vigna radiata* L. Wilczek) were obtained from a local market. 3-Mercaptopicolinic acid was a gift from Dr. N. DiTullio, Smith Kline and French Laboratories. All other biochemicals and auxiliary enzymes were purchased from Sigma, except for PFP which was purified from potato tubers according to Van Schaftingen and Hers (23). Bio-Gel P-6DG was from Bio-Rad.

Measurement of Fru-2,6-P_2. Fru-2,6-P_2 was measured in extracts from individual seedlings. The endosperm tissue (0.8–0.9 g fresh weight) was prepared and killed within 3 min of harvest. To study the effects of anoxia on the content of Fru-2,6-P_2, each endosperm was suspended in a 250-ml flask equilibrated with N_2 at 25°C. For 30 min prior to adding the endosperm, and

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[2] Present address: Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada.

[3] Abbreviations: Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; 3-PGA, 3-phosphoglycerate; FBPase, fructose 1,6-bisphosphatase (EC 3.1.3.11); PFP, pyrophosphate:fructose 6-phosphate phosphotransferase (EC 2.7.1.90); Fru-6-P,2K, fructose 6-phosphate 2-kinase; Fru-2,6-P_2ase, fructose 2,6-bisphosphatase.

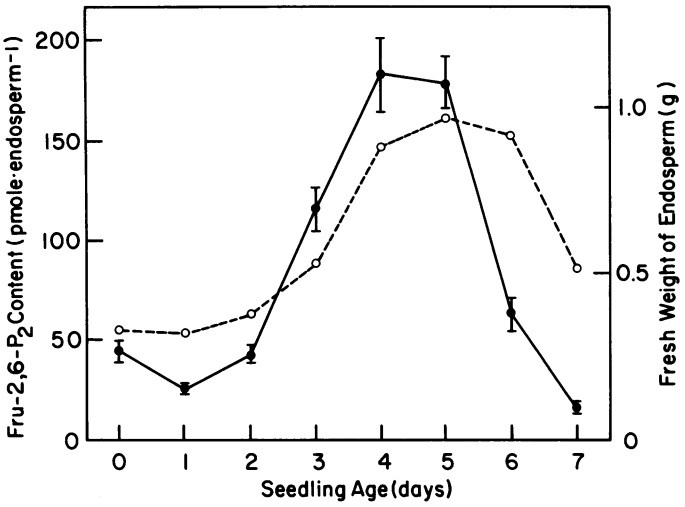

FIG. 1. Changes in Fru-2,6-P_2 in endosperm during early growth. Fru-2,6-P_2 (●) was measured in extracts of freeze-clamped tissue at the times indicated. Each value represents the mean ± SE of four separate samples. The mean fresh weight of endosperm (○) is included for reference.

Table I. *Effect of Anoxia on Fru-2,6-P_2 Content of Endosperm*

Endosperm isolated from 4-d-old castor bean was incubated in either air or N_2 for 60 min at 25°C before being freeze-clamped, killed, and extracted. Control samples were freeze-clamped within 1 min after being detached from the seedlings. Fru-2,6-P_2 was measured in these extracts by activation of PFP. Each value is the mean ± SE of four separate samples.

Treatment	Fru-2,6-P_2 Content
	pmol·endosperm^{-1}
Control	169.1 ± 23.5
Air, 60 min	163.3 ± 8.2
N_2, 60 min	315.2 ± 28.5

during the experiment, the flasks were continuously flushed with moist N_2 (200 ml/min). Each sample was killed about 2 s after removal from N_2. For treatment with 3-mercaptopicolinic acid, endosperm halves were placed on a filter paper (7 cm diameter) in a Petri dish. The filter was moistened with 1 ml 5 mM Mes-NaOH (pH 5.25) containing 2.5 mM 3-mercaptopicolinic acid and the upper surface of each endosperm half was treated with 100 μl of the same solution. The samples, each containing two endosperm halves, were then incubated at 25°C for 150 min before being killed.

For extraction, all endosperm samples were freeze-clamped, as described in apRees *et al.* (1), between two aluminum blocks (70 cm^2 cross-sectional area, 1.5 cm thick) that had been precooled in liquid N_2. The frozen, powdered tissue was extracted exactly as described in Stitt *et al.* (20), except that the extract was finally redissolved in 1 ml H_2O, and was not treated with activated charcoal.

Fru-2,6-P_2 was assayed by its activation of PFP from potato tubers (23). The reaction mixture contained, in 1 ml, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM Fru-6-P, 0.1 mM NADH, 0.5 mM PPi, 0.03 IU (when fully activated) PFP, 1 IU aldolase, 10 IU triosephosphate isomerase, 1 IU glycerol-3-phosphate dehydrogenase, and up to 20 μl extract. The assay was started by adding PPi after 5 min preincubation. The amount of Fru-2,6-P_2 in each sample was calculated by comparing the extent of activation with that induced by known amounts of authentic Fru-2,6-P_2. Fru-6-P used in this assay was checked for contamination by Fru-2,6-P_2 (11).

Measurement of Enzyme Activity. For measurement of maximum catalytic activities, endosperm from five seedlings was homogenized with a mortar and pestle, and then with an all-glass homogenizer, in about four volumes of 100 mM triethanolamine-HCl (pH 7.7), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 2% (w/v) PVP. Microscopic examination of the homogenate revealed very few unbroken cells. The homogenate was centrifuged at 30,000*g* for 20 min and a 1-ml portion of the supernatant was passed through a column (1 × 7 cm) of Bio-Gel P-6DG equilibrated with extraction buffer from which PVP was omitted. The desalted extract was used for enzyme determinations.

Fru-6-P,2K was measured as described in Cséke and Buchanan (2). The reaction mixture contained, in a total volume of 100 μl, 100 mM Hepes-NaOH (pH 7.5), 10 mM Fru-6-P, 23 mM glucose 6-phosphate, 2 mM ATP, 5 mM $MgCl_2$, 5 mM Pi, 10–20 μl extract and was incubated at 25°C. The reaction was started by adding ATP. At various times aliquots of the reaction mixture were removed and immediately analyzed for Fru-2,6-P_2 by activation of PFP. The sample, usually 50 μl, was added to the PFP assay mixture described above in "Measurement of Fru-2,6-P_2."

Fru-2,6-P_2ase was assayed by two methods (3). The first method was by measuring the production of Fru-6-P spectrophotometrically. The reaction mixture contained, in a total volume of 1 ml, 100 mM Mes-NaOH (pH 6.5), 2 mM Fru-2,6-P_2, 5 mM $MgCl_2$, 1 mM $NADP^+$, 2 IU phosphoglucoisomerase, 1 IU glucose-6-phosphate dehydrogenase, 0.25 IU 6-phosphogluconate dehydrogenase. The reaction was started by adding up to 50 μl extract. Alternatively, Fru-2,6-P_2ase was assayed as described above for Fru-6-P,2K except that the disappearance of Fru-2,6-P_2 was measured. The reaction mixture contained 100 mM Mes-NaOH (pH 6.5), 50 nM Fru-2,6-P_2, 5 mM $MgCl_2$. The reaction was started by adding extract. At appropriate times the Fru-2,6-P_2 content of aliquots of the reaction mixture was determined. Other enzymes were measured as follows: pyrophosphatase (8), phosphofructokinase (10), FBPase (10), aldolase (15), phosphoglucoisomerase (15).

Protein was measured according to Lowry *et al.* (14) after precipitation by 5% (w/v) TCA. BSA was used as a standard.

Determination of Kinetic Constants. Michaelis constants, K_m and V, were obtained by linear regression of v against v/s (Eadie-Hofstee plot). Values for $S_{0.5}$ were calculated from Hill plots.

RESULTS

Authentication of Fru-2,6-P_2 Assay. We checked that the assay for Fru-2,6-P_2 described by Van Schaftingen *et al.* (23) accurately measured this compound in extracts of castor bean endosperm. The potato PFP used in these assays was purified 175-fold with 70% yield (specific activity 11 μmol·min^{-1} mg $protein^{-1}$). The purified preparation was free from detectable activity of phosphofructokinase, aldolase, Fru-2,6-P_2ase, and pyrophosphatase. The activities of FBPase and phosphoglucoisomerase were less than 5 and 10%, respectively, of that of PFP and are unlikely to interfere with the assay.

Several metabolic intermediates are known to affect the response of PFP to Fru-2,6-P_2 (7). To check that such compounds in the endosperm extracts did not interfere with the assay, 1 pmol authentic Fru-2,6-P_2 was added to the reaction mixture containing endosperm extract. The increased stimulation by Fru-2,6-P_2 was from 90 to 108% of the stimulation of PFP in a similar assay from which extract was omitted. Acid treatment of the endosperm extract as described in Kruger *et al.* (11) removed intrinsic Fru-2,6-P_2 and abolished the stimulation of PFP activity. Subsequent addition of authentic Fru-2,6-P_2 gave the anticipated stimulation. The extreme acid lability indicates that stimulation by the castor bean extract was due solely to Fru-2,6-P_2 and not to other sugar phosphates that are also known to activate PFP (8).

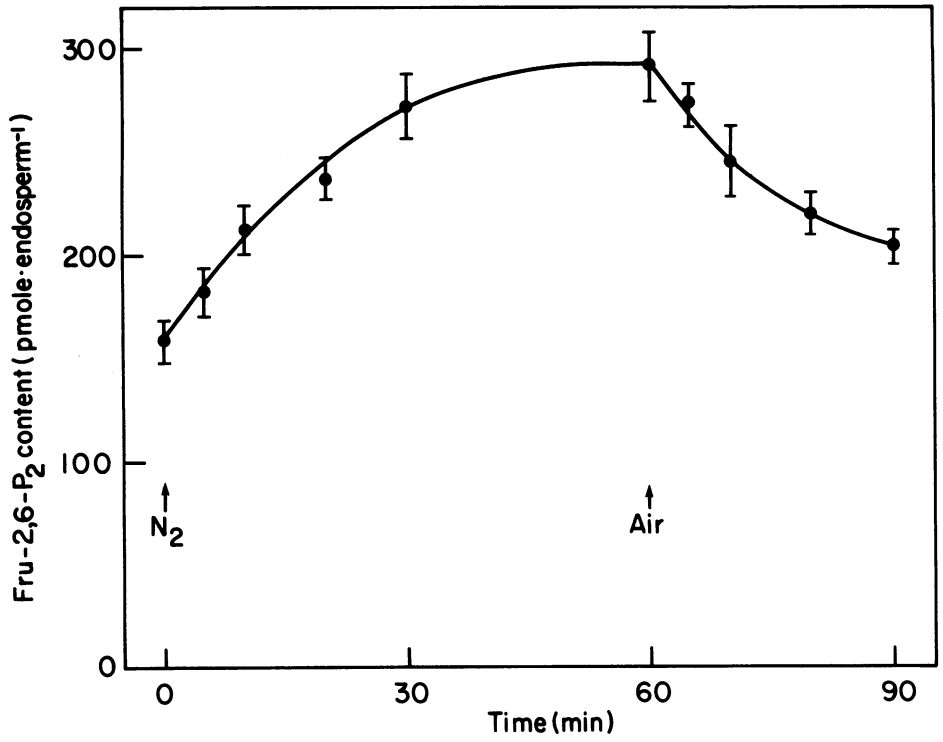

FIG. 2. Changes in the level of Fru-2,6-P_2 in endosperm in transition from air to N_2, and N_2 to air. Endosperm isolated from 4-d-old seedlings was incubated in N_2 for up to 60 min, or N_2 for 60 min and then air as indicated. Each sample was freeze-clamped and killed. Fru-2,6-P_2 was measured in the resulting extracts. Each value represents the mean ± SE of three separate experiments.

Table II. *Effect of 3-Mercaptopicolinic Acid on Fru-2,6-P_2 Content of Endosperm*

Isolated endosperm from 4-d-old castor beans was treated with 5 mM Mes-NaOH (pH 5.25) or 5 mM Mes-NaOH (pH 5.25) that contained 2.5 mM 3-mercaptopicolinic acid. After 150 min at 25°C each sample was freeze-clamped, killed, and extracted. Control samples were not treated with buffer and were freeze-clamped within 1 min after being detached from the seedlings. Fru-2,6-P_2 was measured in each of the extracts. Each value is the mean ± SE of four separate samples.

Treatment	Fru-2,6-P_2 Content
	pmol·endosperm^{-1}
Control	156.7 ± 24.7
Buffer, 150 min	164.8 ± 24.2
3-Mercaptopicolinic acid, 150 min	349.8 ± 20.8

We investigated whether Fru-2,6-P_2 was lost during the killing, extraction, and analysis of endosperm. Duplicate samples of endosperm were freeze-clamped, killed with methanol/chloroform, extracted and analyzed identically, except that 100 pmol Fru-2,6-P_2 was added to the liquid N_2 used to disrupt one of the samples. From a comparison of the two samples, 91 ± 4% (mean ± SE of four separate experiments) of the added Fru-2,6-P_2 was recovered in the final extract. The above results indicate that our measurements of Fru-2,6-P_2 are reliable.

Fru-2,6-P_2 Content of the Endosperm. Estimates of the level of Fru-2,6-P_2 in castor bean endosperm throughout the 7-d period are presented in Figure 1. A small amount of Fru-2,6-P_2 was present at the earliest stages of germination. This amount had increased about 6-fold by the 4th d, when gluconeogenesis was most rapid, before declining rapidly after the 5th d as the endosperm degenerated. This pattern of development is similar to that of several other phosphorylated intermediates (6). The absolute amounts of Fru-2,6-P_2 are less than 1% of those of Fru-1,6-P_2 and Fru-6-P (6). Very low levels of Fru-2,6-P_2 in comparison to other hexose phosphates have been observed previously in spinach leaves (20).

We investigated whether changes in Fru-2,6-P_2 content could contribute to the regulation of gluconeogenesis in the endosperm. Anoxia limits gluconeogenesis and evokes glycolysis in this tissue (6). Under N_2 the level of Fru-2,6-P_2 doubled, whereas the amount of this compound did not significantly increase in tissues left in air (Table I). We studied this change in more detail (Fig. 2). On transfer of endosperm to N_2, the Fru-2,6-P_2 level increased gradually for about 30 min with an initial maximum rate of 5.54 ± 0.27 pmol·min^{-1}·endosperm^{-1} (mean ± SE of three experi-

Table III. *Enzyme Activities in Endosperm during Seedling Development*

Enzyme activities were measured in desalted extracts of endosperm at various times following germination. Fru-6-P, 2K was measured as described in "Materials and Methods." Fru-2,6-P_2ase was measured using the spectrophotometric assay, except that both 2 mM and 5 μM Fru-2,6-P_2 were used. Values are the mean ± SE of four separate extracts.

Seedling Age	Fresh Wt of Endosperm	Protein Content	Enzyme Activity		
			Fru-6-P, 2K	Fru-2,6-P_2ase	
				2 mM Fru-2,6-P_2	5 μM Fru-2,6-P_2
d	*g*	*mg·endosperm^{-1}*	*pmol·min^{-1}·endosperm^{-1}*	*nmol·min^{-1}·endosperm^{-1}*	
2	0.364 ± 0.003	31.55 ± 0.86	30.49 ± 1.64	104.5 ± 9.9	0.590 ± 0.044
4	0.850 ± 0.013	20.25 ± 1.06	57.51 ± 4.66	370.4 ± 9.3	2.348 ± 0.124
7	0.570 ± 0.021	2.77 ± 0.51	27.29 ± 2.41	114.6 ± 7.7	0.637 ± 0.032

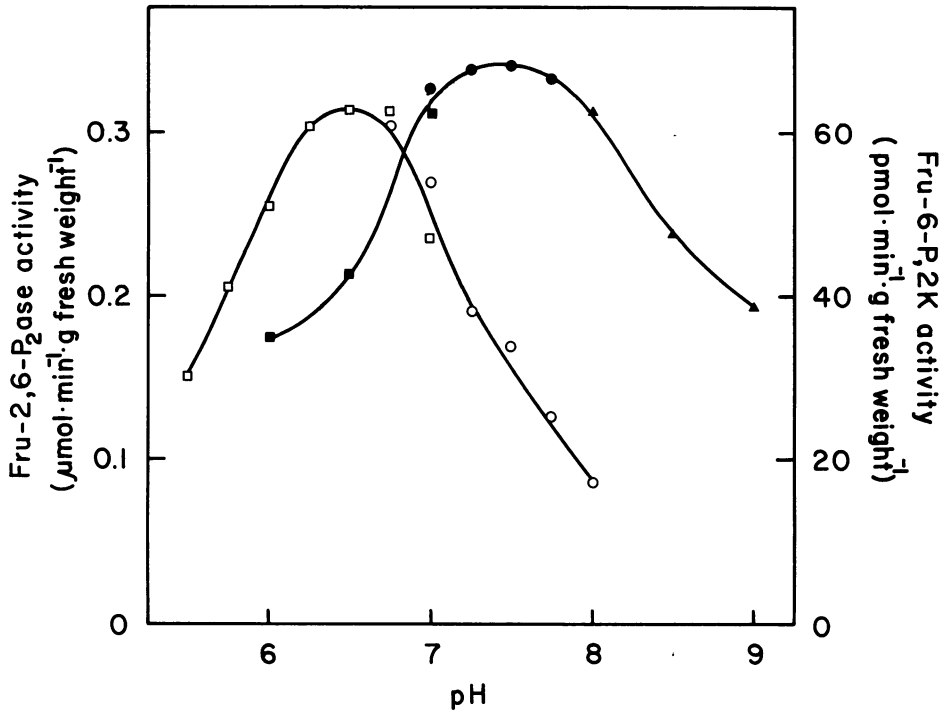

FIG. 3. Effect of pH on enzyme activity. Enzymes were measured in desalted extracts. Fru-6-P,2K (●, ■, ▲) was assayed using 5 mM Fru-6-P and 2 mM ATP. Fru-2,6-P_2ase (○, □) was measured spectrophotometrically using 0.5 mM Fru-2,6-P_2. Buffers, each at 100 mM, were Mes-NaOH (■, □), Hepes-NaOH (●, ○), and Tris-HCl (▲).

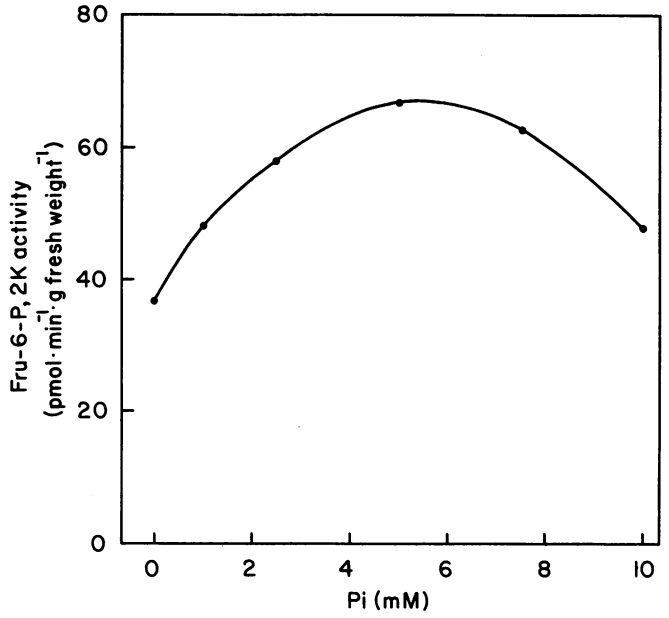

FIG. 4. Effect of Pi on Fru-6-P,2K activity. Fru-6-P,2K was measured using 5 mM Fru-6-P and 1 mM ATP. Pi was varied as shown.

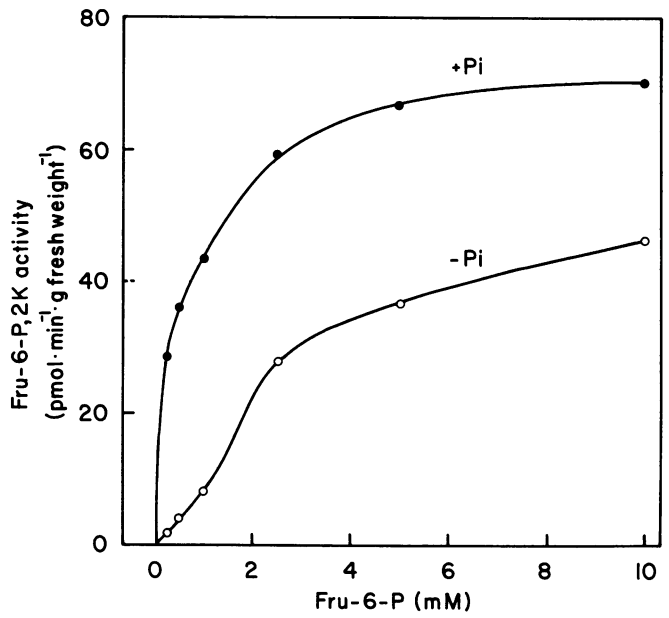

FIG. 5. Effect of Fru-6-P on Fru-6-P,2K activity. Fru-6-P,2K was measured in the presence of 5 mM Pi (●) and in its absence (○). ATP was 1 mM and Fru-6-P was varied as shown.

ments). This rate was maintained for the first 10 min. When endosperm was returned to air after 60 min in N_2 the amount of Fru-2,6-P_2 decreased to a level similar to that present before anoxia. Again, the initial, most rapid decline of Fru-2,6-P_2 was linear for about 10 min at a rate of 6.53 ± 0.33 pmol·min^{-1}·endosperm^{-1} (mean ± SE of three experiments).

To further test the role of Fru-2,6-P_2, we treated endosperm with 3-mercaptopicolinic acid, a potent inhibitor of phosphoenolpyruvate carboxykinase (12). This inhibitor is known to reduce gluconeogenesis in germinating seeds by restricting the production of phosphoenolpyruvate from oxaloacetate (13). Under such conditions the amount of Fru-2,6-P_2 increased to about 250% of the control level (Table II).

Activities of Fru-6-P,2K and Fru-2,6-P_2ase in Endosperm. The changes in the amount of Fru-2,6-P_2 in endosperm, both developmentally and in response to experimental manipulation, indicate that the tissue is capable of synthesizing and degrading this sugar bisphosphate. Estimates of the activities of Fru-6-P,2K and Fru-2,6-P_2ase in the endosperm at various stages of development are presented in Table III. The activities of both enzymes are sufficient to account for the highest rates of Fru-2,6-P_2 synthesis and degradation measured in Figure 2. We checked that the assays gave optimum activity by varying the concentration of each component, and the pH, of the reaction mixture for each developmental stage. Because the amount of Fru-2,6-P_2 required for maximum activity of Fru-2,6-P_2ase is much greater than current estimates of the Fru-2,6-P_2 concentration in plants,

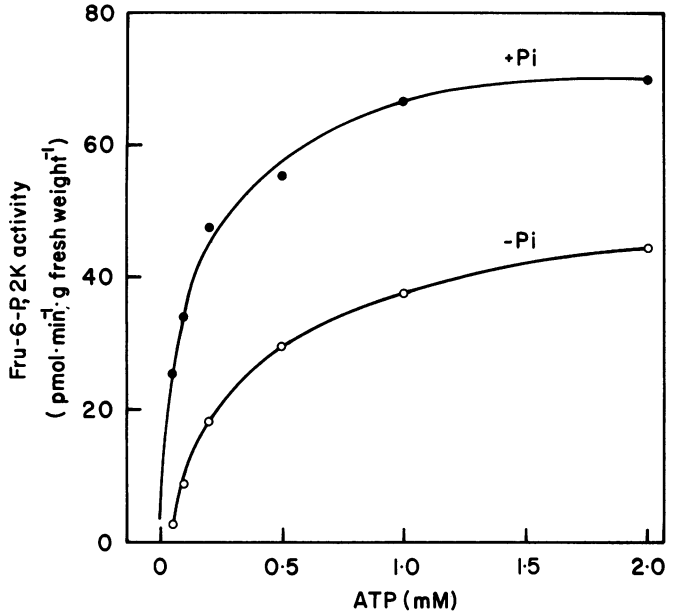

FIG. 6. Effect of ATP on Fru-6-P,2K activity. Fru-6-P,2K was measured in the presence of 5 mM Pi (●) and in its absence (○). Fru-6-P was 5 mM and ATP was varied as shown.

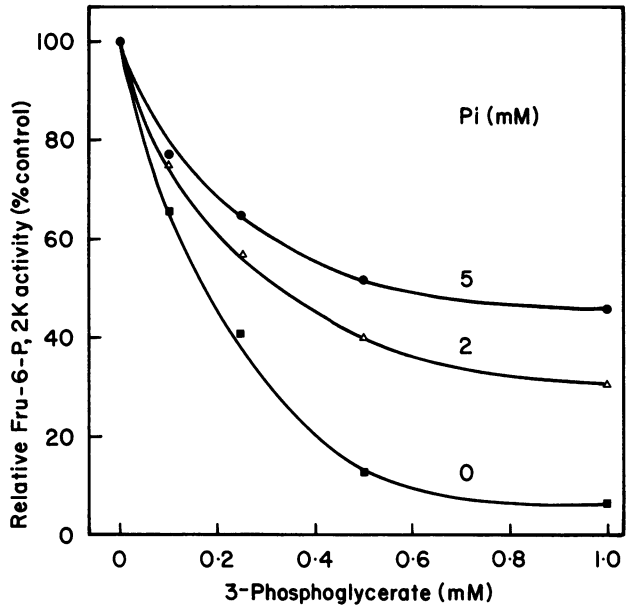

FIG. 7. Effect of Pi on the inhibition of Fru-6-P,2K by 3-PGA. Fru-6-P,2K was measured using 5 mM Fru-6-P, 1 mM ATP, and 3-PGA as shown. The results at each concentration are expressed as a percentage of the activity in the absence of 3-PGA. These activities were 66.7, 55.3, and 36.8 pmol·min^{-1}·g fresh weight^{-1} in the presence of 5 mM (●), 2 mM (△), and no (■) Pi, respectively.

Table III also includes measurements of this enzyme in 5 μM Fru-2,6-P_2, a more physiological substrate concentration (20, 21). To investigate whether the measurements were affected by enzyme inhibitors or activators we measured the activities in a sample of endosperm from 4-d-old plants, a sample of etiolated mung bean hypocotyls, and a sample of equal weights of endosperm and hypocotyl. Under the conditions used the activities of Fru-6-P,2K and Fru-2,6-P_2ase in mung bean hypocotyl were about 25 pmol·min^{-1}·g fresh weight^{-1} and 50 nmol·min^{-1}·g fresh weight^{-1}, respectively. For Fru-6-P,2K the activity found in the mixture was 106 ± 10% (mean ± SE of three samples) of

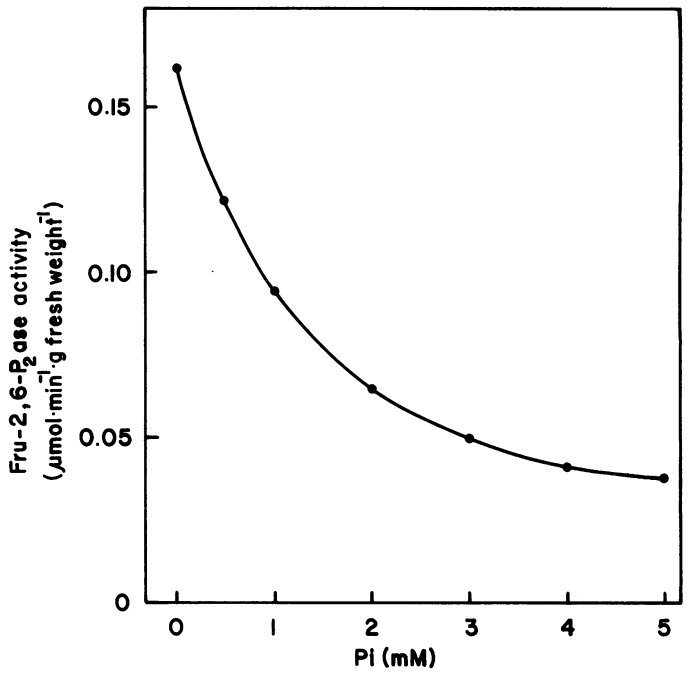

FIG. 8. Effect of Pi on Fru-2,6-P_2ase activity. Fru-2,6-P_2ase was measured spectrophotometrically using 0.5 mM Fru-2,6-P_2. Pi was varied as shown.

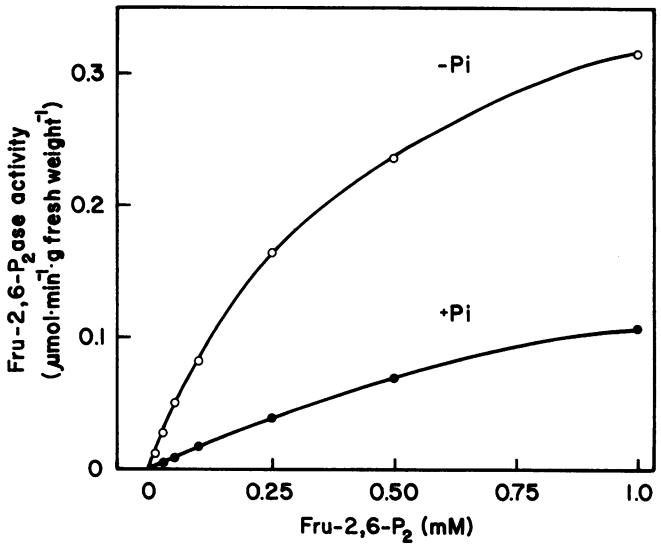

FIG. 9. Effect of Fru-2,6-P_2 on Fru-2,6-P_2ase activity. Fru-2,6-P_2ase was measured spectrophotometrically in the presence of 5 mM Pi (●) or in its absence (○). Fru-2,6-P_2 was varied as shown.

that predicted from measurements made on the tissues extracted separately. The corresponding value for Fru-2,6-P_2ase was 112 ± 8%. Similar values were obtained with endosperm from 2- and 7-d-old seedlings. These results demonstrate that the measurements in Table III represent maximum catalytic activities.

The estimates of Fru-2,6-P_2ase activity are unlikely to be due to a nonspecific phosphatase. Although endosperm extracts catalyzed the release of Pi from *p*-nitrophenylphosphate, this activity was optimum at below pH 5.5 and was not significantly affected by up to 10 mM Pi or by 5 mM EDTA. These properties contrast strikingly with those of Fru-2,6-P_2ase described below.

Properties of Fru-6-P,2K. Fru-6-P,2K exhibited a broad pH optimum with maximum activity at about pH 7.5 (Fig. 3). As found for the spinach leaf enzyme (2), Pi markedly stimulated

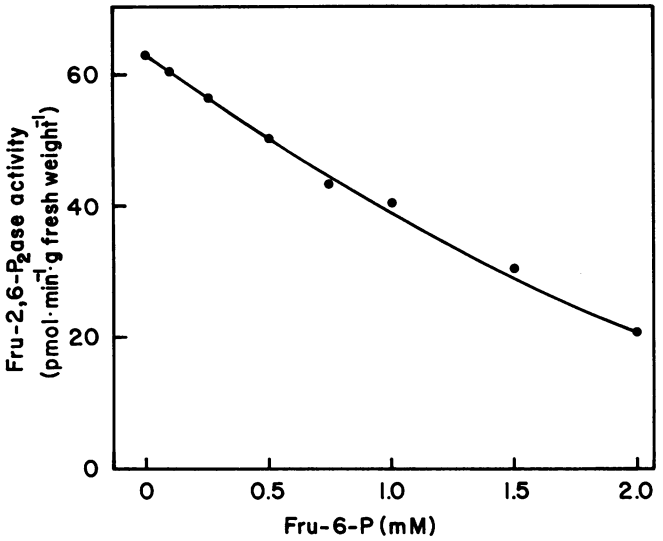

FIG. 10. Effect of Fru-6-P on Fru-2,6-P_2ase activity. Fru-2,6-P_2ase was assayed by measuring the disappearance of Fru-2,6-P_2 (PFP activation bioassay). Fru-2,6-P_2 was 50 nM and Fru-6-P was varied as shown.

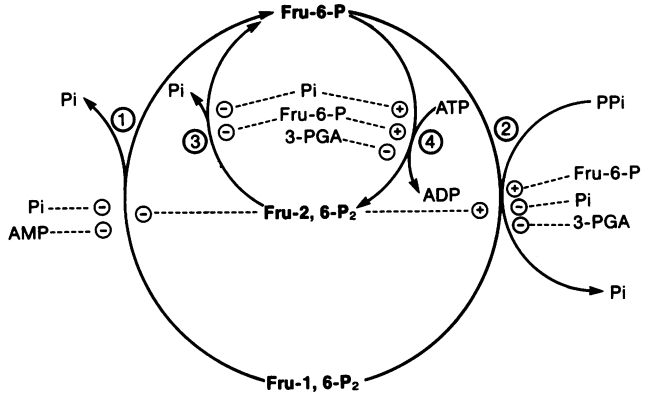

FIG. 11. Summary of the interacting components in the regulation imposed by Fru-2,6-P_2. The numbered reactions are catalyzed by the following enzymes: (1), FBPase; (2), PFP; (3), Fru-2,6-P_2ase; (4), Fru-6-P,2K. Major activators (+) and inhibitors (−) of the enzymes are shown.

Fru-6-P,2K from castor bean endosperm. Maximum stimulation was achieved at 5 mM Pi, and above this concentration of Pi the activity declined (Fig. 4). Pi acts by increasing the affinity for both Fru-6-P and ATP, and by increasing V^{app}. In the absence of Pi the enzyme exhibited sigmoid kinetics with respect to Fru-6-P, but on addition of Pi the kinetics were hyperbolic and $S_{0.5}$ for Fru-6-P was decreased from 2.5 to 0.4 mM (Fig. 5). With respect to ATP, Pi caused a similar decrease in $S_{0.5}$ from 0.38 to 0.10 mM (Fig. 6). In both instances, 5 mM Pi increased V^{app} from 50 to 70 pmol·min^{-1}·g fresh weight^{-1}. In addition, Pi was partially able to relieve the strong inhibition of the enzyme by 3-PGA (Fig. 7).

Properties of Fru-2,6-P_2ase. Optimum activity of Fru-2,6-P_2ase was obtained at pH 6.5 (Fig. 3). This activity was at least partially dependent on Mg^{2+} since, in the absence of added $MgCl_2$, 5 mM EDTA decreased activity about 50%. In direct contrast to Fru-6-P,2K, Fru-2,6-P_2ase was inhibited by Pi. With 0.5 mM Fru-2,6-P_2, 50% inhibition was produced by 1.3 mM Pi (Fig. 8). Again, Pi acted by affecting both the affinity of the enzyme for the substrate and the maximum activity (Fig. 9). In the presence of 5 mM Pi, K_m for Fru-2,6-P_2 was increased 5-fold, from 0.37 to 1.9 mM, while V decreased from 0.41 to 0.33 μmol·min^{-1}·g fresh weight^{-1}. Fru-6-P, the other product of the reaction, also inhibited Fru-2,6-P_2ase with 50% inhibition at about 1.4 mM Fru-6-P (Fig. 10). These latter assays were performed at a much lower Fru-2,6-P_2 concentration so that the disappearance of the substrate during the reaction could be accurately measured.

Because of the apparent reciprocal effect of Pi on Fru-6-P,2K and Fru-2,6-P_2ase we investigated the effect of 3-PGA on the latter enzyme. Up to 2.5 mM 3-PGA did not stimulate Fru-2,6-P_2ase activity between 25 μM and 1 mM Fru-2,6-P_2, nor did it relieve the inhibition by 1 or 5 mM Pi over a similar range of substrate concentrations. Fru-2,6-P_2ase from spinach leaves was also unaffected by 3-PGA (3).

Based on the present results and those from earlier studies (7–9), we present a summary scheme in Figure 11 to facilitate discussion.

DISCUSSION

The results demonstrate that, at all stages, castor bean endosperm contains Fru-2,6-P_2 and the enzymes responsible for its synthesis and degradation. The properties of Fru-6-P,2K from endosperm are very similar to those described for the enzyme from spinach leaf (2). In particular, both enzymes exhibit sigmoid kinetics with respect to Fru-6-P, are strongly activated by Pi, and are inhibited by 3-PGA. The inhibition of endosperm Fru-2,6-P_2ase by both of its products also resembles the properties of the corresponding enzyme from spinach leaf (3). This supports our argument that such activity in castor bean endosperm is not due to a nonspecific phosphatase. However, spinach leaf Fru-2,6-P_2ase apparently has a far greater affinity for Fru-2,6-P_2 and a slightly higher pH optimum than reported for the enzyme in the present study.

Interruption of gluconeogenesis, either by anoxia or treatment with 3-mercaptopicolinic acid, doubles the amount of Fru-2,6-P_2 in the endosperm. This increase is consistent with the proposed regulatory role of Fru-2,6-P_2 *in vivo* (Fig. 11). Previous work on castor bean endosperm has shown that the amounts of other compounds also change at the onset of anoxia (6). The content of 3-PGA which is in equilibrium with 2-phosphoglycerate and phosphoenolpyruvate, decreases markedly and the hexose phosphate levels drop rapidly to between 25 and 30% of their aerobic levels when the tissue is placed in N_2. The decreases in these phosphorylated intermediates, and in ATP (6), presumably produce a corresponding increase in the Pi level. These changes in metabolic intermediates at the beginning of anoxia take about 15 to 30 min to occur, which is similar to the time taken for the peak increase in Fru-2,6-P_2 (Fig. 2), and for the glycolytic flux to be established (6). Similar changes in 3-PGA and Fru-6-P were observed when endosperm was treated with 3-mercaptopicolinic acid as in Table II. After 150 min in contact with 3-mercaptopicolinate the amount of 3-PGA had declined by 71% and that of Fru-6-P by 69%; incubation in buffer alone induced small increases in both metabolites.

The metabolic intermediates described above could contribute to the regulation of Fru-2,6-P_2 level *in vivo* by their effects on the enzymes that synthesize and degrade Fru-2-6-P_2. When gluconeogenesis is restricted Fru-6-P,2K may be stimulated by the increase in Pi and decrease in 3-PGA, while Fru-2,6-P_2ase is inhibited by the elevated Pi level. The decrease in Fru-6-P, which will tend to counteract these responses, is probably insufficient to override their effect. Overall, these changes would result in an increase in the level of Fru-2,6-P_2.

From these results and those of our earlier reports (7–9) we suggest that Fru-2,6-P_2 is probably an important regulator of gluconeogenesis in castor bean. Through its reciprocal effects on the activities of cytoplasmic FBPase and PFP operating in the glycolytic direction, Fru-2,6-P_2 can effectively control the conversion of Fru-1,6-P_2 to Fru-6-P. The level of Fru-2,6-P_2, in turn is probably regulated by several metabolic intermediates, which

may also directly affect both FBPase and PFP as depicted in Figure 11. This scheme would allow the flux from Fru-1,6-P_2 to Fru-6-P to respond to changes in the levels of metabolites, and could coordinate this conversion with the supply of gluconeogenic substrate during germination. These suggestions may require modification as our knowledge of the properties of the enzymes concerned and our understanding of the effects of Fru-2,6-P_2 increase. Stitt *et al.* (19) have recently proposed a similar role for Fru-2,6-P_2 in coordinating sucrose synthesis with the rate of CO_2 fixation in leaves. These workers suggest that in photosynthetic tissues Fru-2,6-P_2 may effectively regulate cytoplasmic FBPase by monitoring the supply and removal of the 3-carbon and 6-carbon intermediates on either side of this reaction. Our results are consistent with this view and extend the idea to the regulation of carbohydrate metabolism in nonphotosynthetic tissues.

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