

## Development and Properties of Fructose 1,6-Bisphosphatase in the Endosperm of Castor-Bean Seedlings

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1. The activity of fructose 1,6-bisphosphatase (EC 3.1.3.11) in the fatty endosperm of castor bean (*Ricinus communis*) increases 25-fold during germination and then declines. The developmental pattern follows that of catalase, a marker enzyme for gluconeogenesis in this tissue. 2. The enzyme at its peak of development was partially purified, and its properties were studied. It has an optimal activity at neutral pH (7.0–8.0). The apparent  $K_m$  value for fructose 1,6-bisphosphate is  $3.8 \times 10^{-5}$  M. The activity is inhibited by AMP allosterically with an apparent  $K_i$  value of  $2.2 \times 10^{-4}$  M. The enzyme hydrolyses fructose 1,6-bisphosphate and not ribulose 1,5-bisphosphate or sedoheptulose 1,7-bisphosphate. 3. Treatment of the partially purified enzyme with acid leads to an 80% decrease in activity. The remaining activity is insensitive to AMP and has optimal activity at pH 6.7 and a high apparent  $K_m$  value ( $2.5 \times 10^{-4}$  M) for fructose 1,6-bisphosphate. Enzyme extracted from the tissue with water instead of buffer has a similar modification. The effect of acid explains the discrepancies between this report and previous ones on the properties of the enzyme in this tissue. 4. The storage tissues of various fatty seedlings all contain a 'neutral' fructose 1,6-bisphosphatase. The activities of the enzyme from some of the tissues are inhibited by AMP. 5. The properties of the enzyme in fatty seedlings and in green leaves are discussed in comparison with that in animal tissues.

During germination of fatty seeds, the reserve lipid is rapidly converted into sucrose (Beever, 1969). Phosphoenolpyruvate derived from triglyceride is converted into hexose by the enzymes of the glycolytic pathway. In the reverse flow of glycolysis, fructose 1,6-bisphosphatase (EC 3.1.3.11) catalyses the hydrolysis of fructose bisphosphate to fructose 6-phosphate, thus by-passing the irreversible step of phosphorylation of fructose 6-phosphate catalysed by fructose 6-phosphate kinase (EC 2.7.1.11). Fructose 1,6-bisphosphatase is thus an important enzyme in the process of gluconeogenesis. The enzyme in animal tissues has been purified and well studied (Marcus *et al.*, 1973; Colombo & Marcus, 1973; Uyeda & Luby, 1974; Kratowich & Mendicino, 1974; review by Pontremoli & Horecker, 1971). It has an optimal activity at neutral pH and its activity is regulated by the allosteric inhibitor AMP. It is a versatile regulatory enzyme since its properties can be modified by metabolites, proteinases and various inorganic ions. A similar enzyme has been found in many microorganisms (Springgate & Stachow, 1972; review by Pontremoli & Horecker, 1971). In fatty seedlings of higher plants where gluconeogenic activity is high, the fructose 1,6-bisphosphatase has not been studied intensively. The enzyme was partially purified from the fatty endosperm of castor bean during germination (Scala *et al.*, 1968*a,b*). It has an optimal activity

at pH 6.7 and its activity is insensitive to AMP. Thus the allosteric nature of the enzyme and its regulatory role in glycolysis in this tissue is still not clear.

We now report experiments on the partial purification and characterization of the fructose 1,6-bisphosphatase from the fatty endosperm of castor bean at the peak stage of germination. The properties of the enzyme in this tissue, such as its sensitivity toward AMP and the apparent  $K_m$  value for the substrate, are quite different from those reported previously (Scala *et al.*, 1968*a,b*). We provide evidence to show that the properties of the enzyme reported previously are not those of the native enzyme, owing to the effect of cellular acid in the endosperm during the extraction procedure.

### Experimental

#### Biochemicals

Purified glucose 6-phosphate dehydrogenase was obtained from Calbiochem (La Jolla, CA, U.S.A.). Fructose 1,6-bisphosphate, fructose 1-phosphate, fructose 6-phosphate,  $\alpha$ -glycerophosphate, glucose 6-phosphate, glucose 1-phosphate, ribulose 1,5-bisphosphate, sedoheptulose 1,7-bisphosphate, bovine serum albumin and phosphoglucose isomerase, all of the purest grades, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### Plant materials

Castor bean (*Ricinus communis* var. Hale), watermelon (*Citrullus vulgaris* Schrad.), corn (*Zea mays*), cucumber (*Cucumis sativus* L.), peanut (*Arachis hypogaea* L.) and sunflower (*Helianthus annuus* L.) were soaked overnight in running tap water, and germinated in moist vermiculite at 30°C in darkness. Spinach (*Spinacia oleracea* L.) leaves were obtained from a local store. The extraction procedure was performed at 4°C. The tissues were first chopped to small pieces with an onion chopper (castor-bean endosperm and spinach leaves) or with razor blades (the rest) in grinding medium and then ground with a mortar and pestle (Huang & Beevers, 1971). The grinding medium contained 0.4 M-sucrose, 1 mM-EDTA, 10 mM-KCl, 1 mM-MgCl<sub>2</sub>, 2 mM-dithiothreitol and 0.15 M-Tricine [*N*-tris(hydroxymethyl)methylglycine] buffer adjusted with KOH to pH 7.5. The homogenate was passed through eight layers of cheesecloth and centrifuged at 270 g for 10 min. The supernatant fractions were used for enzyme assay directly (castor-bean endosperm) or first desalted (the rest) with a column of Sephadex G-25 equilibrated with the grinding medium. For the isolation of chloroplasts, the crude homogenate from spinach leaves was centrifuged at 1000 g for 10 min, and the pellet, which consisted of intact and broken chloroplasts (Rocha & Ting, 1970), was resuspended with the grinding medium.

### Assays

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Catalase activity was assayed spectrophotometrically (Lück, 1965). Fructose 1,6-bisphosphatase activity was assayed either by the reduction of NADP<sup>+</sup> or by the release of P<sub>i</sub>. The assay of NADP<sup>+</sup> reduction was a modification of that described by Racker (1965). The standard reaction mixture contained 0.2 M-Tris/HCl buffer, pH 7.0, 5 mM-MgCl<sub>2</sub>, 3 mM-EDTA, 0.25 mM-NADP<sup>+</sup>, 0.15 unit of glucose 6-phosphate isomerase (EC 5.3.1.9), 0.3 unit of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and enzyme preparation. The reaction was initiated with 1 mM-fructose 1,6-bisphosphate. The standard reaction mixture for the assay of fructose 1,6-bisphosphatase activity by the P<sub>i</sub> method consisted of 0.2 M-Tris/HCl buffer, pH 7.0, 5 mM-MgCl<sub>2</sub>, 3 mM-EDTA and enzyme preparation in a final volume of 1 ml. The reaction was initiated with fructose 1,6-bisphosphate. Portions (0.2 ml) of reaction mixture were mixed with 0.05 ml of 40% (w/v) trichloroacetic acid at various times. The samples were further acidified with 0.2 ml of 82% trichloroacetic acid and then mixed with 0.2 ml of a solution containing 0.013 M-(NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.5 M-H<sub>2</sub>SO<sub>4</sub> and 0.18 M-FeSO<sub>4</sub>·7H<sub>2</sub>O. The absorbance was measured at 660 nm after 10 min (Wharton & McCarty, 1972). The P<sub>i</sub> method was used only for the test of substrate specificity.

### Purification procedure

All steps were carried out at 0–4°C. Castor-bean endosperm (100 g; 5 days old) was ground with 200 ml of grinding medium as described. The homogenate was passed through eight layers of cheesecloth and centrifuged at 10000 g for 30 min. The supernatant fraction obtained was referred to as the crude extract. It was adjusted to 46.5% (w/v) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> by slowly adding solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (64.8 g/225 ml). The precipitate was discarded after centrifugation at 10000 g for 10 min and the supernatant fraction was adjusted to 62% (w/v) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (24.4 g/240 ml). The precipitate was obtained by centrifugation at 10000 g for 10 min and resuspended with 5 ml of 5 mM-sodium malonate buffer, pH 6.0 (referred to as ammonium sulphate fraction). It was applied to a Sephadex G-25 column (1.5 cm × 18 cm) equilibrated with 5 mM-sodium malonate buffer, pH 6.0. The opaque eluate was collected, centrifuged at 10000 g for 30 min, and the supernatant fraction was applied to a Sephadex G-200 column (1.5 cm × 80 cm). The column was eluted with the same buffer, and the fractions (3.0 ml each) from the peak of enzyme activity were pooled.

### Results

The total fructose 1,6-bisphosphatase activity in the fatty endosperm of castor bean increases some 25-fold after 5 days of germination and then declines (Fig. 1). This developmental pattern of fructose 1,6-bisphosphatase activity follows closely that of the catalase activity which has been used as a marker for the gluconeogenesis process in the fatty seedling (Beevers, 1969). The occurrence of maximal enzyme activity in the 5-day-old seedlings is similar to that observed previously (Marx, 1967), but is in slight disagreement with the finding of Osmond *et al.* (1975) who reported that the fructose 1,6-bisphosphatase had its peak activity at around 3–4 days after germination. By organelle fractionation as described earlier (Huang, 1975), more than 90% of the fructose 1,6-bisphosphatase activity was found in the soluble fraction, suggesting that the enzyme occurs in the cytosol rather than in organelles.

The fructose 1,6-bisphosphatase in the 5-day-old endosperm was purified 44-fold by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation and Sephadex-gel chromatography (Table 1). The partially purified enzyme preparation was used to study the properties of the enzyme. A double-reciprocal plot of enzyme activity against substrate concentration in the presence of various AMP concentrations is shown in Fig. 2. The apparent *K<sub>m</sub>* value for fructose 1,6-bisphosphate is calculated to be 3.8 × 10<sup>-5</sup> M. The enzyme activity is inhibited by AMP, which acts as an allosteric inhibitor, as suggested in the double-reciprocal plot. The apparent *K<sub>i</sub>* is estimated to be 2.2 × 10<sup>-4</sup> M. The optimal activity

of the enzyme preparation ranges from pH 7.0 to 8.0 (Fig. 3).

The specificity of the enzyme preparation toward fructose 1,6-bisphosphate and other phosphorylated sugars was tested at two different substrate concentrations (Table 2). At 1.0 mM substrate concentration, which is the optimal concentration of fructose 1,6-bisphosphate, the enzyme also acts on ribulose 1,5-bisphosphate and sedoheptulose 1,7-bisphosphate with activity 34 and 21% respectively of that on fructose 1,6-bisphosphate. The enzyme does not hydrolyse other phosphorylated sugars tested. Ribulose 1,5-bisphosphate and sedoheptulose 1,7-bisphosphate used in the experiments were obtained from Sigma. As reported in the specifications from the Company, the sedoheptulose 1,7-bisphosphate contains approx. 1% of fructose 1,6-bisphosphate as contaminant, and the ribulose 1,5-bisphosphate contains 13.6% of unspecified phosphorylated com-

pounds. Since the enzyme has a low apparent  $K_m$  value for fructose 1,6-bisphosphate ( $3.8 \times 10^{-5} M$ ), the activity observed with ribulose bisphosphate or sedoheptulose bisphosphate may actually be that on the contaminating fructose bisphosphate (1% of fructose bisphosphate in 1 mM-sedoheptulose bisphosphate yields  $10 \mu M$ -fructose bisphosphate). To resolve the problem of contamination of fructose bisphosphate in the two phosphorylated sugars, the concentration of phosphorylated sugars used to assay for activity was lowered to 0.1 mM. At this concentration, although the enzyme still shows activity with fructose bisphosphate similar to that when assayed with 1.0 mM concentration, it has little activity with sedoheptulose bisphosphate or ribulose bisphosphate. Even if no contamination by fructose bisphosphate occurs in the two phosphorylated sugars obtained commercially, the finding suggests that the enzyme has a much lower affinity for sedoheptulose bisphosphate or ribulose bisphosphate than fructose bisphosphate. The evidence is thus clear that the enzyme is specific for fructose bisphosphate.

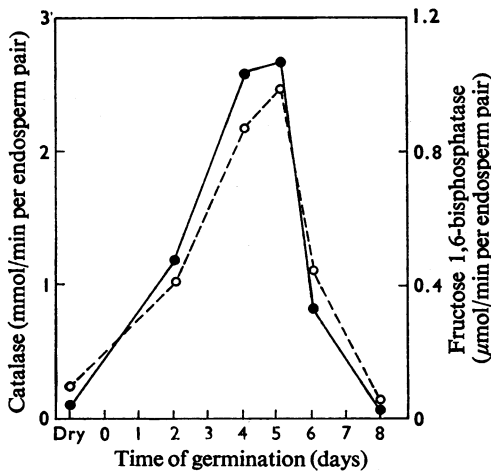


Fig. 1. Changes in the total activities of fructose 1,6-bisphosphatase and catalase in the endosperm of castor bean during germination

●, Catalase activity; ○, fructose 1,6-bisphosphatase activity.

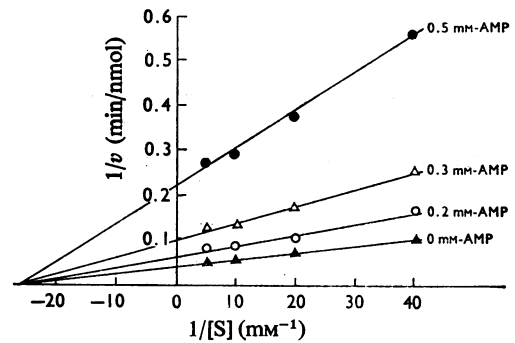


Fig. 2. Double-reciprocal plot of the activities of fructose 1,6-bisphosphatase partially purified from castor-bean endosperm as a function of concentration of fructose 1,6-bisphosphate in the presence of various concentrations of 5'-AMP

For details see the text.

Table 1. Partial purification of fructose 1,6-bisphosphatase from 5-day-old castor-bean endosperm

Fraction	Total activity (μmol/min per 100 g fresh wt.)	Specific activity (μmol/min per mg of protein)	Recovery (%)	Inhibition by 1 mM-AMP (%)	Purification (fold)
Crude extract	94	0.021	—	85	1
Ammonium sulphate (46.5–62%-satd.)	100	0.122	—	85	6
Sephadex G-25	140	0.273	100	91	13
Sephadex G-200	106	0.924	76	90	44

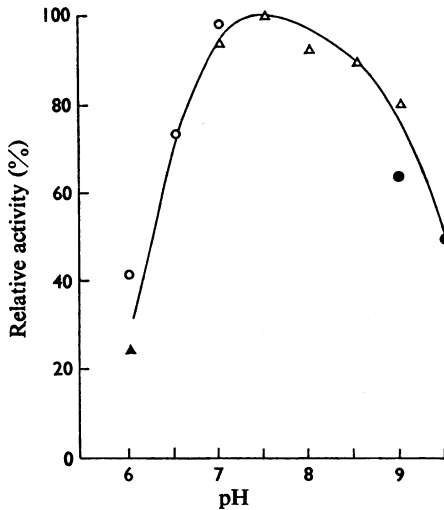


Fig. 3. Effect of pH on the activity of fructose 1,6-bisphosphatase partially purified from 5-day-old castor-bean endosperm

▲, Malonate/NaOH buffer; ○, imidazole/HCl buffer; △, Tris/HCl buffer; ●, glycine/NaOH buffer. 100% relative activity is equivalent to 8.0nmol/min of enzyme activity.

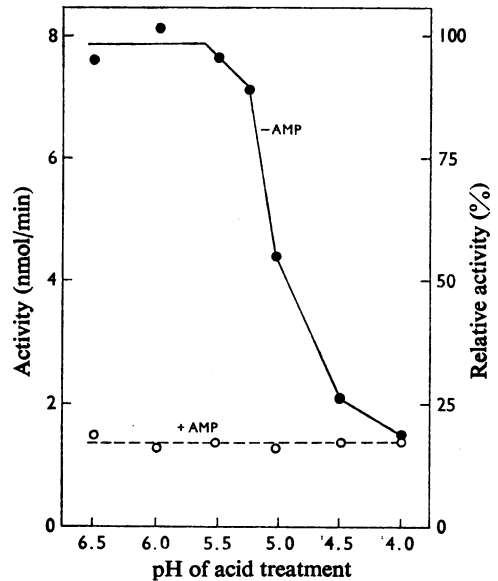


Fig. 4. Effect of acid treatment on the activity of fructose 1,6-bisphosphatase partially purified from 5-day-old castor-bean endosperm

The partially purified enzyme was incubated with an equal volume of 1M-malonate/NaOH for 5min and the enzyme activity was checked in the presence (○) or absence (●) of 1mM-5'-AMP.

Table 2. Substrate specificity of fructose 1,6-bisphosphatase partially purified from 5-day-old castor-bean endosperm

Similar activity (8.0nmol/min) was obtained with fructose 1,6-bisphosphate at either 1.0mM or 0.1mM. Dash denotes activity not checked. Ribulose 1,5-bisphosphate and sedoheptulose 1,7-bisphosphate obtained from commercial source might contain fructose 1,6-bisphosphate as contaminant (see the text for explanation).

Substrate	Substrate concn.	Relative activity	
		1.0mM	0.1mM
Fructose 1,6-bisphosphate		100	100
Ribulose 1,5-bisphosphate		34	6
Sedoheptulose 1,7-bisphosphate		21	3
Fructose 1-phosphate		8	—
Fructose 6-phosphate		0	—
Glycerophosphate		1	—
Glucose 6-phosphate		0	—
Glucose 1-phosphate		0	—

The activity of the partially purified enzyme is sensitive to acid treatment. Brief treatment with an equal volume of 1M-sodium malonate buffer, pH5.0, eliminates 82% of the enzyme activity. The remaining activity has a pH optimum at 6.7 and an apparent  $K_m$  value for fructose bisphosphate of  $2.5 \times 10^{-4}$ M, and it is completely insensitive to AMP

inhibition. As shown in Fig. 4, the condition of the acid inactivation is pH-dependent, and completion occurs at pH4.0 in 5min. Prolonged treatment (1h) of the enzyme preparation at pH5.0 gives a similar effect. It is not known whether the acid treatment brings about a modification of the enzyme molecule including the catalytic capacity as well as the allosteric site for AMP, or whether it eliminates completely the AMP-sensitive fructose bisphosphatase, leaving behind a contaminating phosphatase of low activity in the preparation.

A fructose bisphosphatase from 3-day-old castor-bean endosperm was partially purified previously (Scala *et al.*, 1968a,b). This enzyme, as compared with the one reported in the present study, has a 10-fold higher apparent  $K_m$  value for fructose bisphosphate and a slightly lower pH for maximal activity, and its activity is completely insensitive to AMP inhibition. The discrepancy can be explained by our finding of the sensitivity of the partially purified enzyme preparation toward acid treatment. During the germination of castor bean, organic acids gradually accumulate inside the cells of the endosperm (Kobr & Beevers, 1971), presumably in a certain subcellular compartment. Thus homogenization of the endosperm with water will yield an acidic homogenate. The acidity will eliminate the AMP-sensitive fructose bisphos-

Table 3. *Properties of fructose 1,6-bisphosphatase from 5-day-old castor-bean endosperm after various treatments*

Treatments by acid, added externally or derived from the tissue, modified the properties of the enzyme.

Source	pH optimum	$K_m$ (fructose bisphosphate) (M)	Inhibition by 1 mM-AMP (%)
Buffer extracted, crude	7.5	$3.2 \times 10^{-5}$	85
Buffer extracted, partially purified	7.5	$3.8 \times 10^{-5}$	90
Buffer extracted, partially purified followed by acid treatment	6.7	$2.5 \times 10^{-4}$	8
Water extracted, crude	6.5	$2.6 \times 10^{-4}$	10
Partially purified enzyme reported previously	6.7	$3.3 \times 10^{-4}$	0

Table 4. *Fructose 1,6-bisphosphatase from various fatty seedlings and spinach-leaf chloroplasts*

The activities were assayed at pH 7.0 for all seedlings and at pH 8.5 for spinach-leaf chloroplasts. For further details see the text.

Tissues	Activity ( $\mu$ mol/min per g fresh weight)	Inhibition by 1 mM-AMP (%)	Activity at pH 7.0 Activity at pH 8.5
Castor-bean endosperm (5-day-old)	0.69	85	1.2
Sunflower cotyledon (4-day-old)	0.32	60	1.5
Peanut cotyledon (4-day-old)	0.29	49	1.6
Watermelon cotyledon (4-day-old)	0.54	11	1.7
Corn scutella (4-day-old)	0.35	9	1.6
Cucumber cotyledon (4-day-old)	0.73	0	5.6
Spinach-leaf chloroplasts	2.3	0	0.019

phatase, as demonstrated in the present study by using the partially purified enzyme. It is therefore essential to homogenize the endosperm with buffered grinding medium instead of water. In the previous reports (Scala *et al.*, 1968*a,b*), the enzyme was extracted with water from the endosperm, and the properties of the enzyme obtained are very similar to our acid-treated enzyme preparation, including the apparent  $K_m$  value for fructose bisphosphate, the optimal pH for activity and the insensitivity toward AMP inhibition (Table 3). We tried to compare the enzyme extracted with either buffered grinding medium or water in the same proportion of 1 g of endosperm per 4 ml of medium as described in the previous reports (Scala *et al.*, 1968*a,b*). When the endosperm was ground in buffered grinding medium, the fructose bisphosphatase in the crude extract has the same properties as those of our partially purified enzyme (Table 3). However, when the endosperm was ground in water, the fructose bisphosphatase in the crude extract has the same properties as those of the castor-bean enzyme described previously (Scala *et al.*, 1968*a,b*), and also of our acid-treated enzyme preparation (Table 3). The pH in the crude extract was found to be 5.0. Further, the activity in the crude homogenate extracted with water is only 17% of that with buffered grinding medium. The evidence is thus clear that the enzyme partially purified in the present study represents the native enzyme, presumably the one of phy-

siological importance. The enzyme reported previously (Scala *et al.*, 1968*a,b*) is an enzyme of low activity left after the native enzyme has been eliminated or modified by cellular acid in the endosperm during the extraction procedure.

A survey of fructose bisphosphatase in the storage tissues of various fatty seedlings (Table 4) shows that all tissues contain the enzyme with activity high enough to account for the rate of gluconeogenesis *in vivo* during germination. The enzymes from all tissues have higher activity at pH 7.0 than at pH 8.5, indicating that they are 'neutral' rather than 'alkaline' fructose bisphosphatases. The activity of the enzymes from some, but not all, of the tissues is inhibited by AMP. Since the allosteric site for AMP of mammalian fructose bisphosphatase can be easily modified, it is not known whether the AMP-insensitive fructose bisphosphatase activity detected in some of the tissues is that of the native enzyme or of the enzyme modified during the extraction procedure. Data on the fructose bisphosphatase in the chloroplasts of spinach leaves were obtained for comparison (Table 4). As reported previously (Racker & Schroeder, 1958; Smillie, 1960; Preiss *et al.*, 1967), the spinach chloroplast enzyme is an 'alkaline' fructose bisphosphatase at low  $Mg^{2+}$  concentration with activity 50 times higher at pH 8.5 than at pH 7.0, and the activity is completely insensitive to AMP inhibition.

## Discussion

Among various fatty seedlings, the endosperm of castor bean is the one in which the process of gluconeogenesis had been studied most intensively (Beevers, 1969). The control point in the reverse flow of glycolysis was suggested to be at the interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate (Kobr & Beevers, 1971), but the regulatory role of the fructose bisphosphatase had not been established. The present study on the properties of the fructose bisphosphatase suggests the participation of the enzyme at such a control point. On the basis of the data on the amount of AMP in the endosperm (Kobr & Beevers, 1971) and assuming that the cellular AMP is present in the cytosol rather than in the large cell vacuole, which occupies roughly half of the cell volume, the concentration of AMP in the cytosol is estimated to be 0.07 mM. Such a concentration is within the lower range for AMP inhibition on the fructose bisphosphatase activity *in vitro*. Further, it was demonstrated that the amount of AMP in the cell increases 3-fold within 10 min of anoxia (Kobr & Beevers, 1971). It thus seems that the fructose bisphosphatase may indeed be under metabolic regulation by AMP inside the cell.

In the two major plant tissues with active gluconeogenesis, namely the photosynthetic green leaves and the storage tissues of fatty seedlings, the fructose bisphosphatases exhibit properties that differ widely. The leaf chloroplast enzyme has an alkaline pH for optimal activity and its activity is insensitive to AMP inhibition (Racker & Schroeder, 1958; Smillie, 1960; Preiss *et al.*, 1967; present study). This alkaline fructose bisphosphatase is specific for fructose bisphosphate and does not act on ribulose bisphosphate or sedoheptulose bisphosphate. Similarly to the chloroplast enzyme, the castor-bean endosperm fructose bisphosphatase hydrolyses fructose bisphosphate and does not act on ribulose bisphosphate or sedoheptulose bisphosphate. However, the castor-bean endosperm enzyme has its optimal activity at neutral pH and its activity is sensitive to the allosteric inhibitor AMP. In these latter aspects, the castor-bean endosperm enzyme is similar to the native 'neutral' fructose bisphosphatase found in many mammalian tissues. The mammalian enzyme has been well studied and its activity can be finely controlled by the presence of cellular proteinases, AMP, thiol groups, inorganic ions, pyridoxal phosphate and fatty acids (review by Pontremoli &

Horecker, 1971; Colombo & Marcus, 1973; Marcus *et al.*, 1973; Kratowich & Mendicino, 1974; Uyeda & Luby, 1974). It remains to be seen whether or not the castor-bean endosperm fructose bisphosphatase also exhibits regulatory properties as sophisticated as those of the mammalian enzyme.

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