

Pyrophosphate and Fructose 2,6-Bisphosphate Effects on Glycolysis in Pea Seed Extracts¹

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DOUGLAS A. SMYTH, MIN-XIAN WU², AND CLANTON C. BLACK, JR.*
Biochemistry Department, University of Georgia, Athens, Georgia 30602

ABSTRACT

The participation of pyrophosphate-dependent phosphofructokinase (PPI-PFK) in plant glycolysis was examined using extracts from pea seeds (*Pisum sativum* L. cv Alaska). Glycolysis starting with fructose 6-phosphate was measured under aerobic conditions as the accumulation of pyruvate. Pyruvate accumulated in a medium containing PPI and adenosine diphosphate at about two-thirds of the rate in a medium containing adenosine diphosphate and adenosine triphosphate (ATP). The PPI-dependent pyruvate accumulation had the same reactant requirements and sensitivity to glycolysis inhibitors, sodium fluoride, and iodoacetamide, as the well-established ATP-dependent glycolysis. Added fructose 2,6-bisphosphate stimulated both the PPI-dependent pyruvate accumulation and PPI-PFK activity whereas this modulator had no effect on ATP-dependent glycolysis or ATP-PFK. Collectively these results demonstrate a PPI-dependent glycolytic pathway in plants which is responsive to fructose 2,6-bisphosphate.

and ATP-PFK in the same organ or tissue (1, 6, 7, 16, 20), and that high activities of PPI-PFK are found in tissue such as pineapple leaves or castor bean endosperm which are actively undergoing gluconeogenesis (1, 6, 11).

The discovery that fru 2,6-P₂ activates higher plant PPI-PFK (15) is an additional and clearly important factor in assigning metabolic roles for this enzyme. Purified PPI-PFKs from higher plants increase their maximum catalytic activity and substrate affinities in the presence of added fru 2,6-P₂ (11, 15-17, 19-21) in a manner which favors the glycolytic direction of the enzyme. Our present study with pea seed homogenates shows that this classical plant system for demonstrating ATP-driven glycolysis (10) also functions with PPI. Beginning glycolysis with fru 6-P here we will describe some of the properties of a PPI-dependent pyruvate production in a soluble desalted pea seed extract, including a stimulation of pyruvate accumulation by added fru 2,6-P₂. The results support the hypothesis (6) that plant PPI-PFK participates in glycolysis through the use of PPI both as a phosphate donor and as an energy source.

MATERIALS AND METHODS

Chemicals. All auxiliary enzymes for enzyme assays and biochemicals including fru 2,6-P₂ were purchased from Sigma Chemical Co. Lactate dehydrogenase used for pyruvate determinations was a commercial preparation (Sigma L-1254).

Plant Extracts. Dry pea seeds (*Pisum sativum* L. cv Alaska) were milled into a powder and stored at -20°C. Pea seed powder (15 g) was ground with sand in a mortar and pestle which had been chilled with liquid N₂. The ground pea seeds were suspended in medium (45 ml) containing 0.1 M Tris-HCl (pH 8.0), 10% (v/v) glycerol, 2 mM Na₂-EDTA, 1 mM MgCl₂, 5 mM DTT, and 1% (w/v) insoluble PVP. The homogenate was filtered through one layer of Miracloth (Calbiochem-Behring Corp.) and then was centrifuged at 115,000g for 40 min in a Beckman L5-65B Ultracentrifuge using a type 60 Ti rotor and a running temperature of 5°C. The supernatant was filtered through glass wool and protein in the filtrate was precipitated at 4°C by the addition of solid (NH₄)₂SO₄ to 65% saturation. The protein was collected by centrifugation at 20,000g for 15 min. The protein pellet was resuspended in 25 mM HEPES-NaOH (pH 8.0) and 5% (v/v) glycerol and 1-ml portions were desalted on Bio-Gel P-6 (Bio-Rad Laboratories) columns with a 5.5-ml bed volume using the same resuspension medium. The desalted fractions were combined and used for incubations and assays for enzyme activity. In some experiments, as indicated (Fig. 2, Table III), pea seeds were soaked overnight in running tap H₂O and were used directly for homogenate preparation as described above without any storage at -20°C.

Incubation Media for Measuring Glycolysis. Aliquots of desalted pea seed preparation (generally 0.6-1.9 mg protein) were incubated at room temperature (23°C) in 0.5-ml volumes containing 0.1 M HEPES-NaOH (pH 7.0 or 8.0), 10 mM fru 6-P, 1

Pyrophosphate-dependent 6-phosphofructose 1-phosphotransferase (PPI-PFK³) catalyzes the interconversion of PPI and fru 6-P to Pi and fructose 1,6-P₂ in microorganisms and plants (6, 13, 14). This step in carbon metabolism probably is rate limiting for glycolysis (18). With amoeba and bacteria, measurements of inadequate levels of ATP-PFK activity (13, 14) clearly pointed to a glycolytic function for PPI-PFK. In the initial work on plants, 10 to 20 times more PPI-PFK activity than ATP-PFK was found in pineapple leaves and it was postulated that PPI-PFK functioned in the daily glycolytic carbon turnover associated with CAM and that PPI served as an energy source for glycolysis (6). However, the function of PPI-PFK in higher plants is not as clearly established as is the case for unicellular organisms. Thermodynamic considerations of the reactions catalyzed by PPI-PFK have been used both to favor an entirely glycolytic role for the enzyme (13), as well as the alternative suggestion that the enzyme is freely reversible (11). We now know many plants contain high PPI-PFK activity in tissues which have high glycolytic activity (1, 6, 7, 11, 15-17, 19, 20); but also it is evident that many plants contain significant amounts of both PPI-PFK

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² Recipient of a Visiting Grant from the Academia Sinica. Permanent address: Shanghai Institute of Plant Physiology, Academia Sinica, Shanghai, The People's Republic of China.

³ Abbreviations: PPI-PFK, PPI-dependent phosphofructokinase; fru 6-P, fructose 6-phosphate; ATP-PFK, ATP-dependent phosphofructokinase; fru 2,6-P₂, fructose 2,6-bisphosphate.

mm DTT, 2.5 mM MgSO₄, 1 mM NAD⁺, 16 mM KCl, 1 mM K-Pi, 0.6 mM ADP, and 1 mM Na-PPi. In some experiments, components of the incubation medium were omitted and in others fructose 2,6-P₂, NaF, iodoacetamide, or 1 mM Na-ATP were added as indicated in the text. The pH of the incubation medium was varied (Fig. 2) using a series of 0.1 M Tris-Mes buffers. The incubation was terminated and protein precipitated by the addition of 0.5 ml of 0.9 N HClO₄ and chilling of the solution to 4°C. The solution was clarified by centrifugation for 1 min in a model B Beckman Microfuge and stored at -20°C.

Pyruvate Determinations. A 0.5-ml volume of the deproteinized incubation solution was neutralized by the addition of 300 μl 0.5 N HEPES-KOH (pH 7.5) and 30 μl 10 N KOH, and the resulting perchlorate was removed from solution by centrifugation for 1 min in the model B Beckman Microfuge. Pyruvate content was determined enzymically (4) by the addition of 2 units of lactate dehydrogenase to a 0.5-ml volume containing 0.13 M HEPES-KOH (pH 7.5), 0.2 mM NADH, and usually 150 μl of sample. Any change in *A* at 340 nm associated with the addition of lactate dehydrogenase was measured by using a volume of H₂O equivalent to the sample in the pyruvate assay, and then subtracting this value from all experimental determinations. The amount of pyruvate was calculated from the amount of NADH oxidized and each incubation component was tested for interference in the assay. Recovery was approximately 80% when known amounts of pyruvate were included in the glycolysis incubations.

Assays for Enzyme Activity. Enzyme activities of desalted pea seed extracts (68–750 μg protein) were assayed at 23°C. Activities of P_i-PFK, ATP-PFK, and fructose 2,6-bisphosphatase were determined as described previously (16) with the exception that 2.5 mM MgSO₄ replaced 2.5 mM MgCl₂ in the assay medium. Phosphoglycerokinase was assayed in the gluconeogenic direction (3). The reaction medium (0.5 ml) contained 0.1 M HEPES-KOH (pH 7.5), 1 mM DTT, 1 mM phosphoglycerate, 2.5 mM MgSO₄, 4 units of glyceraldehyde 3-P dehydrogenase, and 1 mM Na-ATP or 1 mM Na-PPi to initiate the reaction. Pyruvate kinase was assayed in the glycolytic direction (5). The reaction medium (0.5 ml) contained 0.1 M HEPES-KOH (pH 7.5), 1 mM phosphoenolpyruvate, 2.5 mM MgSO₄, 40 mM KCl, 0.16 mM NADH, 2 units of lactate dehydrogenase, and 1 mM Na-ADP or K-Pi. One μM fructose 2,6-P₂ was added to all enzyme assays except fructose 2,6-bisphosphatase after a linear reaction rate was obtained.

Protein Determination. The protein content of pea seed preparations was determined according to a dye-binding technique (2) using BSA as the standard.

RESULTS

Characteristics of Pyruvate Production by Pea Seed Extracts.

Pyruvate was synthesized and accumulated when pea seed extracts were incubated under aerobic conditions in a P_i-containing medium suitable for glycolysis (medium given in "Materials and Methods"). The rate of pyruvate accumulation for the pea seed extract was greatest during the 1st h of incubation in the complete medium (Fig. 1). The complete medium containing P_i supported pyruvate production at a rate which averaged, in five experiments, 66% of the rate obtained in the complete medium containing ATP instead of P_i. Although pyruvate may not accumulate as an end product of plant glycolysis *in vivo*, its initial rate of accumulation of 2 nmol/min·mg protein (Fig. 1) in our aerobic incubations was similar to the optimal rates of ethanol production observed by Hatch and Turner (10) in an anaerobic ATP-based glycolysis system.

Figure 1 illustrates that the time course of pyruvate synthesis in the complete media initially is essentially linear before declining. We cannot confidently extrapolate the data in Figure 1 linearly to zero since we, in theory, expect glycolysis to have a

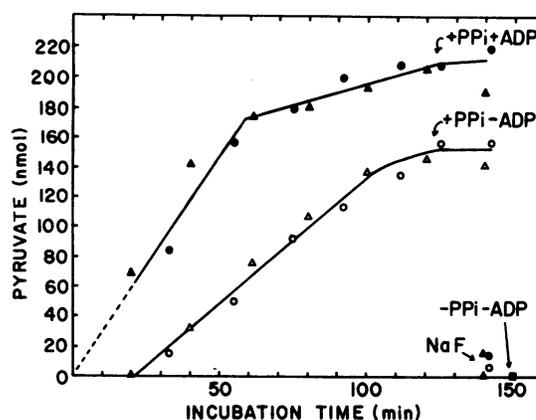


FIG. 1. Time course of pyruvate accumulation in the presence and absence of pyrophosphate, ADP, and NaF. Data are taken from experiments using the complete incubation conditions described in "Materials and Methods." Two mM NaF was included in some of the complete media and medium minus ADP incubations taken to the final time point. The experiments used 1.3 mg pea seed protein for each incubation point. Different symbols represent separate experiments.

Table I. Pyrophosphate-Dependent Pyruvate Accumulation in Incubation Media Lacking Various Components

Aliquots of desalted pea seed extracts, 860 μg protein, were incubated at pH 8.0. The heat-treated pea extract was placed in 98°C H₂O for 10 min prior to adding to the complete incubation medium.

Incubation Component Omitted	Pyruvate Accumulation	% of Complete Medium
	<i>nmol pyruvate/150 min</i>	
Complete	166	100
DTT	148	89
P _i	143	86
PPi	130	78
ADP	73	44
fru 6-P	2	1
Mg ²⁺	0	0
NAD ⁺	0	0
PPi + ADP	1	0
Heat-treated pea extract added	0	0

time lag in the formation of intermediates between fructose 6-P and pyruvate. Certainly the classical glycolysis pathway as studied by Turner and Hatch (10) starting either with fructose or glucose 1-P and ending with CO₂ and ethanol shows about a 1- to 2-h time lag before reaching a linear rate. We are studying a shorter segment of glycolysis, *i.e.* only fructose 6-P to pyruvate, but one still would expect a time lag for pyruvate synthesis.

The general medium requirements to support P_i-driven glycolysis were similar to those found earlier for the ATP-driven glycolysis (10). Pyruvate accumulation was dependent on fructose 6-P, Mg²⁺, NAD⁺, native protein, and P_i plus ADP (Table I). A slight enhancement of pyruvate accumulation occurred by adding P_i which presumably relates to the P_i requirements of glycolysis. DTT was stimulatory in the experiment in Table I, but this stimulation was not always observed. However, both DTT and P_i are included in other work except as noted.

Omission of either P_i or ADP only partially diminished the accumulation of pyruvate during an incubation time of 150 min (Table I). In medium minus P_i, the kinetics of pyruvate accumulation were similar to those found with complete medium for the 1st h of incubation (data not shown), but after longer incu-

bations, the amount of pyruvate accumulated was 77% (mean of five experiments; also Table I) of the amount accumulated in a complete medium. The energy source, other than ADP, necessary to support glycolysis in these incubations without added PPI was not determined. The earlier observation that added ADP stimulated glycolysis in an incubation containing ATP (10) suggest other explanations exist besides adenylate kinase activity ($2 \text{ ADP} \leftrightarrow \text{ATP} + \text{AMP}$).

Pyruvate accumulation without ADP (Table I) demonstrates that a PPI-dependent glycolysis occurs in pea seed extracts. The lag in pyruvate accumulation in the PPI only medium (Fig. 1) was similar to the 1-h lag in ethanol synthesis observed in an ATP-dependent glycolysis system (10). In 11 experiments, the PPI only (minus ADP) system of pyruvate accumulation averaged 67% of that found with the glycolytic system containing both PPI and ADP. Pyruvate production in the presence of PPI but in the absence of added ADP raised the possibility that all three kinases in the glycolytic pathway may couple to a PPI to Pi interconversion rather than to the more classically established systems involving adenine nucleotides. We will address these questions in the following section on levels of enzyme activities.

Levels of Enzyme Activities in Pea Seed Extracts. To better understand the glycolytic activities in pea seed extracts, we measured several enzyme activities related to these studies. The activity of ATP-PFK was slightly greater than PPI-PFK activity in pea seed extracts (Table II). Only PPI-PFK was stimulated by added fru 2,6-P₂ under the enzyme assay conditions, which also were similar to the conditions used during incubations for pyruvate accumulation. Pea seed extracts had significant fru 2,6-bisphosphatase activity against fru 2,6-P at both the pH 8.0 and at pH 7.0 conditions used for most of our studies on pyruvate accumulation. The activity of adenine nucleotide-dependent phosphoglycerokinase was much greater than either PFK or pyruvate kinase activity (Table II; Fig. 2B), which is consistent with the hypothesis that either PFK or pyruvate kinase could be limiting to glycolysis with these pea seed extracts.

As noted earlier, pyruvate accumulation in the glycolysis incubations without added ADP (Table I) led us to examine the Pi ester requirements of phosphoglycerokinase and pyruvate kinase.

Table II. Levels of Enzyme Activity and the Effects of pH, fru 2,6-P₂, and Different Pi Acceptors and Donors on Enzyme Activity

Phosphofructokinase and fructose 2,6-bisphosphatase assays were conducted in the same experiment and included one preparation where the homogenate was centrifuged at 20,000g for 40 min instead of the usual speed of 115,000g. Data are the average of the number of experiments indicated.

Enzyme Measurement	pH	No. of Experiments	Enzyme Activity	
			Normal assay	Plus 1 μM fru 2,6-P ₂
<i>nmol product/min · mg protein</i>				
PPI-dependent phosphofructokinase	8.0	2	2.5	9.6
	7.0	2	1.2	5.1
ATP-dependent phosphofructokinase	8.0	2	13	13
	7.0	2	9.1	9.2
Fructose 2,6-bisphosphatase	8.0	3	1.3	
	7.0	3	4.8	
Pyruvate kinase				
ADP-dependent	7.5	2	22	22
Pi-dependent	7.5	2	0	0
Phosphoglycerokinase				
ATP-dependent	7.5	1	214	176
PPI-dependent	7.5	1	0.4	0.9

Under the conditions used, Pi would not replace ADP in pyruvate kinase assays and phosphoglycerate kinase activity with PPI was less than 1% of the rate with ATP (Table II). Using an extract from imbibed pea seed, PPI-dependent phosphoglycerokinase activity was 4% of the ATP-dependent activity and a Pi-dependent pyruvate kinase was not detected (data not shown). These results are not compatible with the hypothesis that glycolysis can operate by coupling with a PPI to Pi interconversion, but it is also clear that the pyruvate accumulation associated with a PPI-dependent system lacking added ADP is not due to carryover of adenine nucleotide in some incubation component. Incubations with medium containing ADP without PPI or medium containing PPI without ADP results in pyruvate accumulation, whereas medium without added ADP and PPI did not (Fig. 1; Table I). Therefore, it was possible to demonstrate a PPI-dependent pyruvate accumulation independent of adenine nucleotide which was sensitive to glycolytic inhibitors, but it has not been possible yet to demonstrate the necessary enzymology. Note that originally a slower and unexplained glycolysis in pea seed extracts also was observed in the absence of added ADP or ATP (10).

Effects of pH. Pyruvate accumulation was greatest at alkaline pH values (Fig. 2A). Maximum pyruvate accumulation at such alkaline pH values was an unexpected result because PPI-PFK (Fig. 2B) and pyruvate kinase, the other rate-limiting enzyme of glycolysis, have less alkaline pH optima (see references in 18). The shape of the pH profile for pyruvate accumulation might reflect conditions which are more favorable for other required glycolysis components or, alternatively, conditions which are less favorable for reactions competing for pyruvate; but these possibilities remain to be examined.

Effects of Glycolysis Inhibitors. Pyruvate accumulation was inhibited by classical glycolytic inhibitors (Fig. 3) such as NaF which inhibits enolase and iodoacetamide which inhibits triose phosphate dehydrogenase (10, 18). Pyruvate production was sensitive to both NaF and iodoacetamide in the absence of DTT (Fig. 3) and was sensitive only to NaF in the presence of DTT (Fig. 1).

Effects of Added fru 2,6-P on Pyruvate Production by Pea Seed Extracts. Pyruvate accumulation was enhanced by the

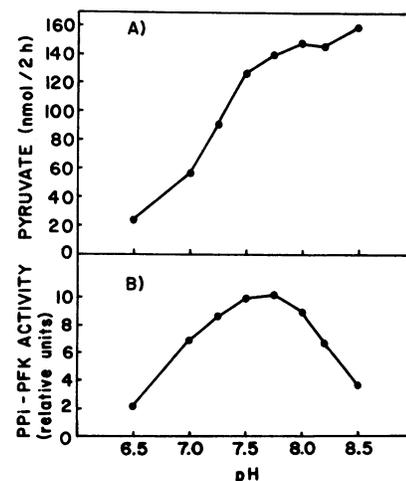


FIG. 2. Effects of pH on PPI-dependent pyruvate accumulation (A) and on PPI-PFK activity (B) using pea seed extracts. Pyruvate accumulation (A) was measured in the complete incubation medium described in "Materials and Methods" using 3.9 mg pea seed protein in each incubation. The pH profile of PPI-PFK activity (B) is shown on a relative basis and is the average of determinations from both dry seed and imbibed seed preparations. The peak specific activity averaged 8.4 nmol fru 6-P/min · mg protein.

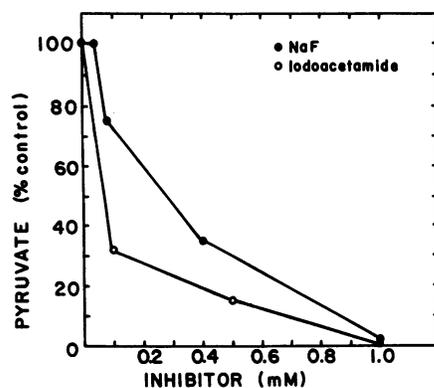


FIG. 3. PPI-dependent pyruvate accumulation with pea seed extracts in the presence of varying concentrations of the glycolytic inhibitors, NaF and iodoacetamide. The incubation time was 106 min and the extract was made from imbibed pea seeds. The control pyruvate accumulation, without inhibitors, was 88 nmol/106 min using 1.2 mg protein. DTT was not added to either incubation medium.

addition of fru 2,6-P₂ (0.2 mM) in a PPI-dependent glycolysis system but not in an ATP-dependent glycolysis system (Table III). In three experiments done at pH 7.0, 0.2 mM fru 2,6-P₂ increased PPI-dependent pyruvate accumulation to 131% (\pm SD of 11%) of the control value whereas the ATP-dependent pyruvate accumulation was not affected (96 \pm 8%). Analysis of variance on the data showed that fru 2,6-P₂-treated samples were statistically different than control samples ($P < 0.01$). In three experiments done at pH 8.0, 0.2 mM fru 2,6-P₂ increased pyruvate accumulation to 112% (\pm 6%) of the control value for the PPI system; this difference was not statistically significant by analysis of variance. Although phosphatase activity against fru 2,6-P₂ was greatest at pH 7.0 (Table II), added fru 2,6-P₂ still stimulated pyruvate accumulation more at pH 7.0 than pH 8.0. At pH 7.0, fru 2,6-P₂ is thought to stabilize PPI-PFK against a dissociation phenomenon (20, 21) which is not observed as readily under more alkaline conditions. Added fru 2,6-P₂ had no effect or was inhibitory when tested at higher concentrations on the PPI-dependent glycolysis system for pyruvate accumulation; e.g. five experiments using 1 to 5 mM fru 2,6-P₂ (data not shown). The effect of 1 to 5 mM fru 2,6-P₂ was also tested on the ATP-dependent system in two of these experiments and these levels inhibited the ATP-dependent glycolysis system more than the PPI-dependent system. Concentrations of fru 6-P equivalent to fru 2,6-P were added to some control incubations to show that additional fru 6-P released by phosphatases did not influence pyruvate accumulation. The reasons for fru 2,6-P₂ inhibition at higher concentrations have not been investigated further.

DISCUSSION

ATP-PFK is a glycolytic enzyme which is irreversible under physiological conditions whereas PPI-PFK is reversible, hence it can theoretically catalyze both glycolytic and gluconeogenic carbon flow (6, 9, 11, 19, 20). Several lines of evidence suggest that interaction between fru 2,6-P₂ and PPI-PFK shifts the catalytic properties of the enzyme towards glycolysis. Both PPI-PFK (1, 8, 11, 16) and the activator fru 2,6-P₂ (15, 17) are in the plant cell cytoplasm. Added fru 2,6-P₂ promotes the association of purified PPI-PFK into a large molecular form with an increased ratio of glycolytic to gluconeogenic activity (20, 21), and in other *in vitro* work, stimulated the glycolytic direction of PPI-PFK at lower fru 2,6-P₂ concentrations than the gluconeogenic direction (19, 20). Because PFK catalyzes the initial step in glycolysis and probably is rate limiting (18), this interaction of PPI-PFK and fru 2,6-P₂ may be an important mechanism for switching plant sugar metabolism between glycolysis and gluconeogenesis (21).

Our present experiments demonstrate that pea seed extracts fed fru 6-P are capable of accumulating pyruvate by a PPI-dependent glycolysis. The conclusion that pyruvate is derived from glycolysis is consistent with the medium requirements for the reaction (Table I; Fig. 1), sensitivity to glycolysis inhibitors (Figs. 1 and 3), and the stimulatory effects of added fru 2,6-P₂ (Table III). Pyruvate accumulated in our PPI-based glycolytic system at a rate which was similar to the rates of ATP-driven ethanol production found by earlier workers (10) also using pea seed preparations. The low level of PFK activity measured in our pea seed preparations (Table II) is consistent with the concept that PFK is one of the limiting steps in glycolysis (18). Furthermore, the stimulation of PPI-PFK and not ATP-PFK by fru 2,6-P₂ (Table II) and the stimulation of PPI-dependent but not ATP-dependent pyruvate accumulation by fru 2,6-P₂ (Table III) also support the idea that PPI-PFK activity is rate limiting in these glycolytic incubations with pea seed extracts. The failure of fru 2,6-P₂ to increase the maximum catalytic activity of plant ATP-PFK has been reported previously (8, 11, 15, 16, 20); in one report with castor bean cotyledons (12), fru 2,6-P₂ increased the substrate affinities of the plastic ATP-PFK but not the maximum velocity. The magnitude of the PPI-dependent glycolysis described herein is significant (about two-thirds) when compared with ATP-driven glycolysis under similar experimental conditions, but the exact quantity of carbon flowing through either pathway remains to be quantitatively established in plants. Recently, we assayed pea and corn tissues for PPI and found the PPI content to be comparable to their ATP content. Collectively, these pea seed data suggest that PPI-PFK is an integral part of a PPI-driven glycolysis in the plant cell cytoplasm which can be

Table III. Effect of fru 2,6-P₂ either on ATP-Dependent or on PPI-Dependent Pyruvate Accumulation

Extracts from imbibed pea seeds were centrifuged at 20,000g for 40 min instead of the usual speed of 115,000g. Incubations were run at pH 7.0. Data represent the mean of duplicate incubations, representing a minimum of four pyruvate determinations.

Phosphate Donor	Experiment No.	Protein	Pyruvate Accumulation		Change
			Standard assay	Plus 0.2 mM fru 2,6-P ₂	
		mg	nmol pyruvate/2 h · mg protein		%
ATP	1	1.87	92	91	-1
	2	0.91	146	129	-12
PPI	1	1.87	59	71	+20
	2	0.91	60	81	+35

stimulated by fru 2,6-P₂.

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