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INORGANIC PYROPHOSPHATE:D-FRUCTOSE-6-PHOSPHATE 1-PHOSPHOTRANSFERASE IN PLANTS AND ITS REGULATION BY A NATURALLY OCCURRING ACTIVATOR, D-FRUCTOSE 2,6-BISPHOSPHATE

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By

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ABSTRACT

INORGANIC PYROPHOSPHATE:D-FRUCTOSE-6-PHOSPHATE 1-PHOSPHO-TRANSFERASE IN PLANTS AND ITS REGULATION BY A NATURALLY OCCURRING ACTIVATOR, D-FRUCTOSE 2,6-BISPHOSPHATE

By

Dario C. Sabularse

Inorganic pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase was detected in extracts of mung bean sprouts, the first such detection in a C_3 plant and in non-photosynthetic plant tissue. The enzyme was purified over 1000-fold. It had an absolute requirement for fructose 6-phosphate and inorganic pyrophosphate as well as for a divalent metal (Mg^{++}), and was found to be activated by hexose bisphosphates (especially by D-fructose 2,6-bisphosphate, but also by D-fructose 1,6-bisphosphate and D-glucose 1,6-bisphosphate), the first demonstration of a regulatory mechanism for this enzyme from any source. It exhibited hyperbolic kinetics both in the absence and presence of a hexose bisphosphate activator. The enzyme was also demonstrated, for the first time, to exist in two forms (large, M_r = 340,000 and small, M_r = 170,000). K_A values of the large and small forms, respectively, for fructose-2,6-P_2, were 25 nM and 50 nM at pH 7.8, the pH optimum, and 25 nM and 140 nM at pH 7.0. In the presence of 1 μM fructose-2,6-P_2, K_m values for fructose-6-P at pH 7.8 were 0.12 mM for both forms, and at pH 7.0 were 0.15 mM and 0.50 nM, respectively, for the large and small forms. The K_m for PP_i was about 0.1 mM for both

forms at both pH values. Fructose 2,6-bisphosphate (1 μM) increased the affinity of the enzyme for fructose-6-bisphosphate 167-fold and increased the V_{max} 15-fold; these two effects combined to give over 1000-fold activation at 0.12 mM fructose-6-phosphate. The enzyme was unaffected by millimolar amounts of phosphoenolpyruvate. In contrast, ATP:D-fructose-6-phosphate 1-phosphotransferase from the same source was not affected by fructose 2,6-bisphosphate but was inhibited >99% by 10 μM phosphoenolpyruvate. Other properties of the two enzymes are also described.

Fructose 2,6-bisphosphate was demonstrated for the first time to be naturally occurring in mung bean and other higher plants. Inorganic pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase was also found to be widespread among plant species. The data support a proposal of this thesis, that the use of inorganic pyrophosphate by this enzyme is instrumental in carbohydrate metabolism in plants, and therefore is of major metabolic significance.

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LIST OF ABBREVIATIONS

Abbreviations used without definition were taken from the list in the Journal of Biological Chemistry Instructions to Authors - 1982. Additional abbreviations used are listed below.

C_3 plant	a plant utilizing ribulose-P_2 for initial fixation of CO_2.
CAM	crassulacean acid metabolism
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediaminetetracetate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MES	2-(N-Morpholino)ethane sulfonic acid
P_i	inorganic orthophosphate
PP_i	inorganic pyrophosphate
PIPES	1,4-piperazinediethane sulfonic acid
mU	milliunit (milliunits)

All sugars are of the D-configuration unless indicated otherwise.

INTRODUCTION

Inorganic pyrophosphate (PP_i) is generated in many major biochemical processes. Among them are amino acid activation during protein synthesis, nucleotide polymerization during nucleic acid synthesis, acyl-CoA formation via acyl-CoA synthetase during activation of fatty acids, and formation of sugar nucleotides during carbohydrate metabolism. It is a generally accepted concept that the PP_i generated in many biochemical systems is hydrolyzed by inorganic pyrophosphatase, thereby providing a favorable thermodynamic condition towards the direction of PP_i formation for the prior reaction. PP_i hydrolysis in this manner dissipates the high energy of the pyrophosphate bond, however, whereas energy would be conserved if the PP_i could be utilized by PP_i-dependent phosphoryl-transfer enzymes, thereby sparing an equivalent ATP while at the same time serving to pull the PP_i-generating reaction.

There are a number of phosphoryl-transfer enzymes, primarily in microorganisms, that are known to use PP_i as the phosphoryl donor. One of these enzymes is inorganic pyrophosphate:D-fructose-6-P 1-phosphotransferase (PP_i:F6P 1-phosphotransferase) which, at the start of this investigation, was known to occur only in microorganisms. Since the reactions involving PP_i formation are ubiquitous in living systems, it was decided to investigate the possible occurrence of the

enzyme in non-photosynthetic tissue of a C_3 plant, in particular sprouted mung beans (Phaseolus aureus). The choice of a germinating starchy seed was prompted by the knowledge that PP_i is formed in large amounts during the conversion of starch to sucrose, that the intracellular localization of inorganic pyrophosphatase is primarily in the chloroplast, and that the tissue would be devoid of photosynthetic processes, which could complicate analysis. In addition, it may be speculated that during the early stages of germination, when the plant is yet at its photosynthetic-independent stage, ATP-sparing reactions would be advantageous.

This thesis will demonstrate (i) the existence and widespread occurrence of PP_i:F6P 1-phosphotransferase in plants, (ii) the first discovery of the activation of this enzyme by hexose bisphosphates, in particular a very potent activation by fructose-2,6-P_2, (iii) the first demonstration of the natural occurrence of fructose-2,6-P_2 in higher plants, (iv) the purification and properties of the enzyme from mung bean sprouts, (v) the first detection of two catalytically active forms of enzyme, and (vi) other findings that are relevant to a proposal that PP_i utilization via PP_i:F6P 1-phosphotransferase is important in energy metabolism in plants.

REVIEW OF LITERATURE

Enzymes That Use Inorganic Pyrophosphate as the Phosphoryl Donor.

There are several phosphotransferases that use PP_i as the phosphoryl donor in at least some organisms (1, 2). They are: (a) acyl--CoA synthetase, EC 6.2.1.1 to EC 6.2.1.3 (ATP + acylate + CoA $\rightleftarrows$ AMP + PP_i + Acyl-CoA), for which in some organisms the net flux could be from right to left (1); (b) phosphoenolpyruvate carboxytransphosphorylase, EC 4.1.1.38 (PP_i + oxaloacetate $\rightleftarrows$ phosphoenolpyruvate + P_i + CO_2), discovered by Siu and Wood (3); (c) glucose-6-phosphatase, EC 3.1.3.9 (glucose + PP_i $\rightleftarrows$ glucose-6-P + P_i), which catalyzes the indicated reaction under certain conditions (4, 5); (d) pyruvate, orthophosphate dikinase, EC 2.7.9.1 (ATP + P_i + pyruvate $\rightleftarrows$ phosphoenolpyruvate + PP_i), which occurs in plants and certain microorganisms (6-9); (e) pyrophosphate:serine phosphotransferase, EC 2.7.1.80 (PP_i + L-serine $\rightarrow$ L-serine-P + P_i), found in propionic acid bacteria (10); (f) PP_i:F6P 1-phosphotransferase EC 2.7.1.90, (PP_i + fructose-6-P $\rightleftarrows$ fructose-1,6-P_2 + P_i), which will be elaborated on in the following section; and (g) PP_i-dependent acetate kinase, EC 2.7.1.2, (acetyl-P + P_i $\rightleftarrows$ acetate + PP_i), present in an anaerobic amoeba (11).

PP_i has been reported to be a product of photophosphorylation in Rhodospirillum rubrum chromatophores (12). In this photosynthetic bacterium, the PP_i is an energy donor for several energy-linked

reactions, including cytochrome reduction (13), transhydrogenation (14), NAD^+ reduction (15), and ATP synthesis (16).

Distribution and Some Properties of PP_i:F6P 1-Phosphotransferase.

Subsequent to the discovery of the enzyme in Entamoeba histolytica (a parasitic, anaerobic amoeba) in 1974 by Reeves and co-workers (17), the enzyme was reported to be present in four bacterial species: Propionibacterium shermanii (18), Bacteroides fragilis (19), Pseudomonas marina (20) and a marine Alcaligenes species (20); and, during the course of this thesis research, in leaves of pineapple, a CAM plant (21). As will be demonstrated in this investigation, the enzyme is now known to be widely distributed in plant species, and in a recent abstract, it was reported to be present in rat liver (22).

The enzyme from all reported sources is specific for PP_i and fructose-6-P. No other mono- or diphosphorylated sugar can substitute for fructose-6-P and no nucleoside triphosphate or polyphosphate can replace PP_i (17, 18, 21). The substrate affinities of the enzyme from E. histolytica and P. shermanii, respectively, are: K_m for fructose-6-P, 38 μM and 100 μM; K_m for PP_i, 14 μM and 69 μM; K_m for fructose-1,6-P_2, 18 μM and 51 μM; and K_m for P_i, 800 and 600 μM. The enzyme isolated from P. shermanii is a dimer of 95,000 molecular weight (18), whereas the E. histolytica enzyme has a molecular weight of 83,000 (23). No regulatory properties have been previously reported for the enzyme from any source.

Discovery, Chemical Synthesis and Identification of Fructose 2,6-Bisphosphate.

The discovery, chemical synthesis, and identification of fructose-2,6-P_2 are credited to the independent but concurrent efforts of three separate laboratory groups: Hers' group - University de Louvain, Brussels (24-28), Pilkis' group - Vanderbilt University, Nashville, Tennessee (29-31), and Uyeda's group - University of Texas, Dallas, Texas (32-36).

Van Schaftingen, Hue, and Hers (24, 25) reported the activation of ATP-dependent phosphofructokinase by a low-molecular-weight effector which could be isolated from rat liver extract by ultrafiltration, gel filtration or heat treatment but was rapidly destroyed in trichloroacetic acid even in the cold. The low-molecular-weight stimulator was a nonreducing derivative of fructose-6-P and was completely destroyed upon incubation with 0.01 M HCl for 10 min at 20°C, with the consequent formation of equimolar amounts of fructose-6-P and P_i. These findings led to the tentative identification of the stimulator as fructose-2,6-P_2. Van Schaftingen and Hers (26) first described a chemical synthesis of the low-molecular-weight stimulator by mixing fructose-6-P with phosphoric acid. However, the method has very low yield and other sugar phosphates are generated. Nevertheless the chemical synthesis experiment proved that the only components of the stimulator were phosphate and fructose-6-P. Employing the method by Pontis and Fischer (37) for the conversion of fructose-1-P to fructose-2-P, a procedure for the chemical synthesis of fructose-2,6-P_2 was worked out by Van Schaftingen and Hers (27). The synthesis involved intramolecular cyclization of fructose-1,6-P_2 to fructose 1,2 cyclic,

6-bisphosphate, followed by alkaline hydrolysis, digestion of the fructose-1,6-P_2 by fructose-1,6-bisphosphatase, and purification of the fructose-2,6-P_2 by column chromatography on Dowex AG1. The chemically synthesized fructose-2,6-P_2 had properties identical to that of the naturally occurring activator from rat hepatocyte extract. Upon examination by ^{31}P and ^{13}C nuclear magnetic resonance spectroscopy, the configuration and structure were deduced to be β-D-fructose 2,6-bisphosphate (28).

From Pilkis' group, evidence for a low-molecular-weight activator from rat hepatocyte extracts for the ATP-dependent phosphofructokinase that was influenced by glucagon was reported (29, 30). The properties of the low-molecular-weight activator were similar to those that had been just reported by Van Schaftingen and Hers for fructose-2,6-P_2 (30). Their subsequent paper (31) reported on the chemical synthesis of fructose-2,6-P_2 from fructose-1,6-P_2 by (i) cyclization utilizing the dicyclohexylcarbodiimide method of Pontis and Fischer (37), to form fructose 1,2 cyclic,6-bisphosphate, (ii) ring opening by alkaline treatment, (iii) heat treatment at alkaline pH to destroy the fructose-1,6-P_2 but not the fructose-2,6-P_2, and (iv) purification by chromatography on a DEAE-Sephadex column. The structure of the chemically synthesized sugar bisphosphate, which had properties identical to that of the activator from hepatocyte extract, was definitively identified as β-D-fructose-2,6-P_2 by mass spectrometry and ^{13}C NMR spectroscopy.

Uyeda's group reported the isolation of an "activation factor" for phosphofructokinase in liver extract in articles by Furuya and Uyeda (32, 33). They noted that the "activation factor" overcomes the ATP

inhibition but had no effect on the catalytic activity under optimum assay conditions for ATP-dependent phosphofructokinase. Furthermore, they observed that AMP acted synergistically with the "activation factor" in reversing ATP inhibition. Richards and Uyeda (34) observed that the "activation factor" for ATP-dependent phosphofructokinase in isolated hepatocytes changed its concentration in response to glucose and glucagon. Glucose increased the concentration of the "activation factor" but glucagon decreased it. In subsequent publications, Uyeda et al. (35, 36) have shown by chemical analysis, synthesis, and ^{13}C NMR spectroscopy that the "activation factor" for the ATP-dependent phosphofructokinase is β-D-fructose-2,6-P_2. The synthetic compound was prepared from fructose-1,2 cyclic,6-P_2 by alkaline hydrolysis and separation by paper chromatography. The synthetic and natural fructose-2,6-P_2 showed identical effects on the allosteric kinetic properties of both rat liver and rabbit muscle phosphofructokinase.

It should be recognized that the three laboratory groups reported practically the same findings and succeeded in an almost simultaneous publication of their work.

Assay of Fructose 2,6-Bisphosphate.

Pure preparations of chemically synthesized fructose-2,6-P_2 can be determined by measuring the amount of fructose-6-P and/or P_i revealed after mild acid hydrolysis (26, 31, 35). In extracts and partially purified samples, fructose-2,6-P_2 is detected by its ability to activate the ATP-dependent phosphofructokinase at sub-optimal substrate concentration (26, 31, 34). For a quantitative determination in tissue extracts, a method has been described by Hue et al. (38), based

on the measurement of the acid-revealed fructose-6-P in a coupled enzymatic assay with bacterial NADH-linked luciferase. Another method is by extraction in hot neutral or alkaline buffers followed by determination of the fructose-2,6-P_2 by comparing the amount of activation of ATP-dependent phosphofructokinase with sample of standard fructose-2,-6-P_2. The activation of homogeneous rat hepatic ATP-dependent phosphofructokinase is found to be roughly linear over the range of 3-10 nM when the enzyme is assayed with 0.2 mM fructose-6-P and 1 mM ATP (39).

Enzymes Regulated by Fructose 2,6-Bisphosphate.

ATP-dependent phosphofructokinase. Fructose-2,6-P_2 is a potent activator of rat liver ATP-dependent phosphofructokinase with K_A = 50 nM. The activation is in the form of increasing the affinity for fructose-6-P but has no effect on the V_{max} of the enzyme (31, 35, 40). The hexose bisphosphate has also been shown to potentiate the activation of ATP-dependent phosphofructokinase by AMP (35, 40, 41), to act synergistically with AMP to release ATP inhibition (35), to release citrate inhibition (42), and to increase the binding affinity of the enzyme to AMP in yeast (43).

ATP-dependent phosphofructokinases from other sources are also activated by fructose-2,6-P_2: rabbit muscle (31, 35), yeast (44, 45), Ehrlich ascites tumor (46) and pancreatic islets of albino rats (47, 48). In higher plants, only the plastid isozyme of ATP-dependent phosphofructokinase from developing endosperm of castor bean has been reported to be activated by fructose-2,6-P_2 when assayed at pH 7.0 with no activation at pH 8.0; the cytosolic isozyme is unaffected at either pH 7.0 or 8.0 (49).

Fructose 1,6-bisphosphatase. This enzyme, which functions at a control point in the gluconeogenic pathway, is also regulated by fructose-2,6-P_2 in rat liver (31, 50-52) and yeast (45). Fructose-2,6-P_2 at low concentration inhibited fructose-1,6-bisphosphatase competitively with the substrate, and also potentiated AMP inhibition of the enzyme (50); at higher concentration it transformed the shape of the substrate concentration-activity curve from hyperbolic to sigmoidal (51).

Enzyme for the Synthesis and Breakdown of Fructose 2,6-Bisphosphate.

The enzyme that catalyzes the synthesis of fructose-2,6-P_2 in rat liver was first reported by Furuya and Uyeda (53), and subsequently by El-Maghrabi et al. (54), Hue et al. (55), and Van Schaftingen and Hers (56). The enzyme catalyzed the transfer of γ-phosphoryl group of ATP to the hydroxyl present in carbon 2 of fructose-6-P and has been given the trivial names of fructose-6-P,2 kinase (53), 6-phosphofructo 2-kinase (54) and phosphofructokinase 2 (56). In this literature review it will be referred to as fructose-6-P,2-kinase. Fructose-6-P,2--kinase is found in liver, brain, heart muscle, kidney, testes and skeletal muscle of rats (57), and in yeast cells (58). The fructose-6-P, 2-kinase has also been shown to be regulated by cyclic AMP-dependent phosphorylation with the resulting activation of the enzyme (59-62).

The enzyme (fructose-2,6-bisphosphatase) responsible for the hydrolysis of the phosphate from the C-2 position of fructose-2,6-P_2 was first reported by Furuya et al. (63). Richards and co-workers (64) observed that glucagon (10^{-11} M), epinephrine (10^{-5} M), or calcium (2.4 mM) and ionophore A23187 (10^{-5} M) administration to hepatocytes produced simultaneous activation of fructose-2,

6-bisphosphatase and inactivation of fructose-6-P,2-kinase within 2 minutes. They suggested that the level of fructose-2,6-P_2 is controlled by reciprocal changes in fructose-2,6-bisphosphatase and fructose-6-P,2-kinase activities.

El-Maghrabi et al. (65) had purified fructose-2,6-bisphosphatase to homogeneity and demonstrated that fructose-6-P,2-kinase activity co-purified with the bisphosphatase activity. They reported that the catalytic subunit of the cAMP-dependent protein kinase phosphorylated the enzyme (1 mole of phosphate/mole subunit of the dimeric enzyme) resulting to a concomitant activation of fructose 2,6-bisphosphatase and an inhibition of the fructose-6-P,2-kinase activity. This phosphorylation-dephosphorylation involved a seryl residue per subunit of the dimeric bifunctional fructose-6-P,2-kinase/fructose-2,6-bisphosphatase (66).

Review Articles on Fructose 2,6-Bisphosphate.

There are two recent reviews on fructose-2,6-P_2. The first is by Pilkis et al., entitled "Fructose 2,6-bisphosphate: a mediator of hormone action at the fructose 6-phosphate/fructose 1,6-bisphosphate substrate" (66a) and the second is by Hers and Van Schaftingen, entitled "Fructose 2,6-bisphosphate 2 years after its discovery" (66b).

Starch Metabolism During Seed Germination.

Starch metabolism has been reviewed extensively by many authors (67-72). However, this literature survey will focus only on starch metabolism during seed germination, particularly for cereals and legumes.

During seed maturation of cereals, starch accumulates as a major carbon-source reserve for eventual carbon-energy needs during germination. In cereal seeds, vigorous synthesis of reserve starch occurs from about six days after flowering to ripening (73). This reserve starch is, however, enzymatically degraded to low-molecular-weight carbohydrates which are further metabolized during the course of germination. In legumes such as soybean, starch is a transient reserve material in both developing and germinating cotyledon (74). During germination of several legume species, amylase activity increases and starch is depleted (75-77) but the seed apparently produces starch during imbibition and germination (78,79).

In nonphotosynthetic organs, the synthesis of reserve starch occurs in amyloplasts, which are a specialized form of plastids that function for starch synthesis (80). Starch occurs in nature as a water-insoluble granule. Its size and shape is often characteristic of the plant species and the plant maturity (81, 82). Starch occurs in two forms. Amylose, the linear type of starch, consists of D-glucose residues in $\alpha(1\text{-}4)$ linkage. Amylopectin, the branched form, consists of chains containing, on the average, about 20-25 $\alpha(1\text{-}4)$-linked D-glucose residues which are interlinked to a branched structure. The molecules thus contain 4-5% of $\alpha(1\text{-}6)$-D-glucosidic linkages (72).

According to Preiss and Levi (68), since starch of plants primarily occurs as water-insoluble granules in distinct plastids, starch degradation must occur in three phases. These are: (i) reduction of the granule to soluble maltodextrins, (ii) debranching and degrading the larger maltodextrins to glucose and glucose-1-P, and (iii) further metabolism of glucose or glucose-1-P and movement (translocation) of

the products from the site of starch storage. The cleavage of α(1-4) bonds of starch may be accomplished by amylases and maltase or by starch phosphorylase, and the α(1-6) bonds by hydrolytic debranching enzymes (71). The pathway and the enzymes involved in the degradation of starch to glucose or glucose-1-P are shown in Figure 1.

The reaction mediated by the various hydrolytic enzymes during starch degradation are well known. A brief description, taken from Preiss and Levi (68) and Manners (72) of the action of these various hydrolytic enzymes is given below.

1. α-Amylases. These enzymes catalyze an essentially random hydrolysis of non-terminal α-(1-4)-glucosidic linkages in both linear and branched substrates. The normal end products are maltose and glucose from amylose and these sugars, together with branched oligosaccharide α-dextrins, from amylopectin. At low enzyme concentration, maltotriose may also be present (72).

2. β-Amylases. This group of enzymes which occur only in cereals and in certain other higher plants such as sweet potatoes and soybeans catalyses a stepwise hydrolysis of alternate linkages starting at the nonreducing end in starch-type polysaccharides with the liberation of maltose. Enzyme action on linear substrates is usually complete; with branched substrates, the enzyme is unable either to hydrolyse or bypass the α-(1-6)-D-glucosidic inter-chain linkages so that the enzyme action is incomplete, the products being maltose and a β limit-dextrin (68, 72).

3. α-Glucosidases. α-Glucosidase hydrolyzes the α(1-4) linkages of dextrins, attacking from the nonreducing end and liberating glucose.

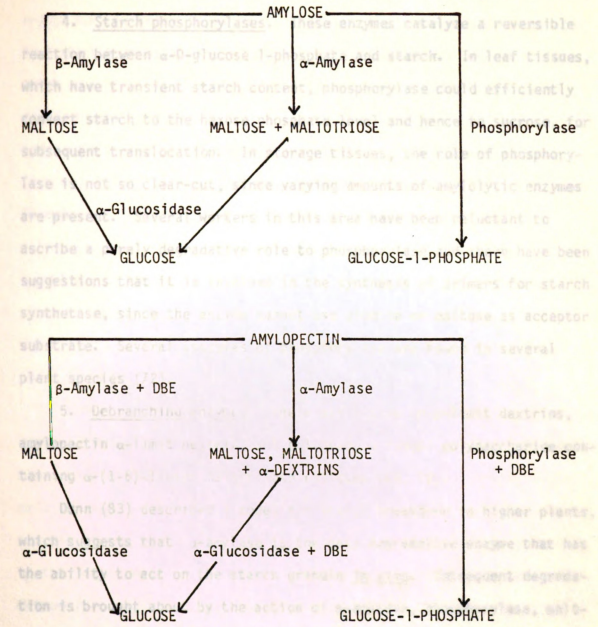

Figure 1. Pathways for the degredation of starch components (DBE = Debranching enzyme). From: Manners, D.J. (1974) In Plant Carbohydrate Biochemistry (Pridham, J.B., ed.) pp. 109-125. Academic Press, London and N.Y.

The α-glucosidases are most active in the hydrolysis of maltose, but they also hydrolyze maltotriose and maltotetrose (68, 72).

4. Starch phosphorylases. These enzymes catalyze a reversible reaction between α-D-glucose 1-phosphate and starch. In leaf tissues, which have transient starch content, phosphorylase could efficiently convert starch to the hexose phosphate level and hence to sucrose, for subsequent translocation. In storage tissues, the role of phosphorylase is not so clear-cut, since varying amounts of amylolytic enzymes are present. Several workers in this area have been reluctant to ascribe a purely degradative role to phosphorylase and there have been suggestions that it is involved in the synthesis of primers for starch synthetase, since the enzyme cannot use glucose or maltose as acceptor substrate. Several isozymes of phosphorylase are found in several plant species (72).

5. Debranching enzymes. These are active on β-limit dextrins, amylopectin α-limit dextrin, and pullulan, a fungal polysaccharide containing α-(1-6)-linked maltotriose residues (68, 72).

Dunn (83) described a model for starch breakdown in higher plants, which suggests that α-amylase is the only degradative enzyme that has the ability to act on the starch granule in vivo. Subsequent degradation is brought about by the action of β-amylase, phosphorylase, maltase and debranching enzymes. It has been pointed out that the initial rate-limiting step in the process of starch degradation is the action of α-amylase on the starch-granule surface.

Starch degradation during germination is paralleled by increase in α-amylase activity. The increase in α-amylase in germinating wheat and barley seeds has been attributed to the de novo synthesis of the enzyme

of the aleurone cells in response to gibberellic acid (84-86). Ho and Varner (87) concluded that there is no accumulation of an inactive α-amylase precursor in barley aleurone cells. Gibbons (88) observed that during germination of barley seeds the α-amylase protein moiety moves away from the entire face of the scutellum and intermediate crushed cell layers. As growth of the seedlings proceeds, the enzyme begins to be synthesized in the aleurone layer and transported away from the layer to the endosperm. Studies by Okamoto et al. (89) and Okamoto and Akazawa (90) on cereal seeds (barley, wheat, rye, oat, maize and rice) in the early stages of germination have shown that α-amylase is synthesized in the epithelial cells of the scutellum. The observation supports the report of Goswami et al. (85) that germinating wheat seeds with intact embryo had greater α-amylase activity than those with embryo excised.

A recent report by MacGregor and Matsuo (91) supports the observations of Okamoto et al. (89) and Goswami et al. (85). They observed by means of scanning electron microscopy that starch degradation, in kernels of wheat and barley during the initial stages of germination, started at the endosperm-embryo junction and moved along the junction to the dorsal edge of the kernel. From these results, MacGregor and Matsuo (91) suggested that the site of initial α-amylase synthesis in germinating cereal grains is in the embryo and not the aleurone layer.

In contrast to de novo synthesis of α-amylase, β-amylase is present in some seeds in the latent form and is transformed to its active form on germination (72, 84, 85). Palmiano and Juliano (92) reported that in germinating rice, increases in α-amylase activity are blocked by protein synthesis inhibitors, but β-amylase activation is not.

However, de novo synthesis of β-amylase in rice scutellum during germination had been reported (93). Okamoto and Akazawa (93) reported that their results indicated strongly that at the onset of germination of rice seeds β-amylase is synthesized de novo in the scutellum and that in the later stages there occurs activation of an inactive, latent form of the enzyme associated with the starch granules in the endosperm which become dominant in later stages of germination.

Phosphorylase, debranching enzyme and α-glucosidase had been found in many mature and in germinating cereal seeds. Phosphorylase has been reported in sweet corn (94), rice (92, 95) and wheat (96). Debranching enzyme has been reported in malted barley (97, 98), sweet corn (99), waxy maize (100) and rice (92). α-Glucosidase has been reported in malted barley (101), rice (102), sorghum (103) and corn (103-105).

Harris (106) studied, by means of electron microscopy, the cotyledon cells of germinating mung bean seeds. He reported that starch grains had shown erosions from within leading to the formation of a hollow shell, and the erosion was accompanied by intrusion of cytoplasm into the shell. He also observed rougher inner surface of the shell suggesting that it was the site of starch hydrolysis; the hydrolyzing enzymes were presumed to be in the cytoplasm for no evidence was found of vesiculation associated with the inner face of the starch shell. The above sequence was for large starch grains which are present in the mature seed prior to germination. These starch grains of germinating mung bean were not surrounded by membranes, nor were there remains of plastid membranes, as in starch grains of germinating Pisum sativum (107). However, during germination cotyledon cells of legumes generally develop plastids which synthesize small compound starch-

grains similar to those reported in Pisum arvense (108). The pattern of breakdown of these membrane-bound starch grains has not been clearly described.

In germinating peas, Juliano and Varner (75), have concluded that α-amylase is the major enzyme involved in the initial degradation of starch into more-soluble forms while phosphorylase and β-amylase assist in the further conversion to free sugars. α-Amylase is synthesized de novo during germination (75, 96, 109, 110). β-Amylase and phosphorylase appear within hours of imbibition (96, 107). Debranching enzyme was found in particles in the ungerminated pea seed (111-113). The enzyme is activated during germination.

In germinating lentils, studies by Tarrago and Nicolas (77) reported increase of α-amylase and phosphorylase very similar to that seen in peas, but β-amylase activity was very low and no mention was made of the debranching enzyme.

Much of the knowledge about starch degradation during seed germination comes from studies with cereals and limited studies with legumes. It appears that there are species variations on the mechanism of the breakdown of starch with respect to the hydrolytic enzymes that predominate. However, it appears that the enzymatic degradation of starch during germination is initiated by α-amylase action. The products are then degraded by α-and β-amylase, phosphorylase, debranching and α-glucosidase activities. The increase in activities of the enzymes is presumably due to de novo synthesis at the onset of germination.

Sucrose Metabolism During Germination of Cereals and Legumes.

Sucrose is the main form of carbohydrate for translocation in nearly all higher plants (114). The nonreducing property of sucrose is regarded to be the important characteristic of a transport species (115). Arnold (116) proposed the hypothesis that sucrose acts as a protective derivative of glucose. If glucose were the translocate species, it would readily be attacked by enzymes which catalyze its metabolism (116).

Several studies have shown that during seed germination, the site of sucrose synthesis is the scutellum. In germinating wheat seeds, glucose was absorbed from the endosperm by the scutellum and synthesized to sucrose in that tissue (117). Similar results were observed in germinating rice (118) and in Avena fatua seeds (119).

By the action of phosphorylase on starch, glucose-1-P is produced. In the conversion of glucose-1-P to sucrose, the first enzyme involved is UDP-glucose pyrophosphorylase which catalyzes the reaction (120) UTP + D-glucose-1-P $\rightleftarrows$ UDP-glucose + PP_i. UDP-glucose pyrophosphorylase was first purified from mung bean seedlings by Ginsburg (121). It was observed that this enzyme was specific for UDP-glucose. Delmer and Albersheim (122) found that UDP-glucose pyrophosphorylase activity is high in extracts of nonphotosynthetic tissue of mung beans.

From UDP-glucose, there are two alternative routes for sucrose formation. One route involves the following reactions: UDP-glucose + fructose-6-P $\rightleftarrows$ UDP + sucrose-6'-P by the action of sucrose phosphate synthase; and sucrose-6'-P $\rightarrow$ sucrose + P_i catalyzed by sucrose phosphate phosphatase. The fructose-6-P is formed from glucose-1-P by the action of phosphoglucomutase and phosphoglucose isomerase. The

second route involves the reaction, UDP-glucose + fructose ⇄ UDP + sucrose, which is catalyzed by sucrose synthase. Leloir and his collaborators (123, 124) discovered the existence of these enzymes capable of synthesizing sucrose and sucrose phosphate.

The generally accepted major route in the synthesis of sucrose in plants is the sucrose phosphate synthase pathway (67, 120, 125). Results of studies by Hawker (126) on germinating broad beans, maize seeds, and castor beans have all suggested that sucrose phosphate synthase and sucrose phosphatase catalyze the synthesis of sucrose via sucrose phosphate. Preiss and Greenberg (127) reported that sucrose phosphate synthase from wheat germ exhibited sigmoidal saturation curves for fructose-6-P and UDP-glucose. It has been observed that Mg^{++} enhances the activity of wheat germ sucrose phosphate synthase in the direction of sucrose synthesis whereas sucrose inhibited its activity (128, 129). The apparent affinity of the enzyme for Mg^{++} was also decreased by sucrose thereby reducing the activity induced by Mg^{++} (129).

Sucrose degradation is achieved by the reversal of the sucrose synthase reaction, sucrose + UDP (ADP) ⇄ UDP-glucose (ADP-glucose) + fructose, which is the principal mechanism of sucrose cleavage in plant cells (125, 130). Studies on sucrose synthase from mung beans showed that the major function of the enzyme is the catalysis of the synthesis of nucleoside diphosphate glucose from translocated sucrose in nonphotosynthetic tissue (131, 132).

Sucrose breakdown is also achieved by the action of invertase. According to Akazawa and Okamoto (133), this breakdown of sucrose is also common in higher plants. The resulting free glucose and fructose

are eventually oxidized to CO_2 and H_2O.

It should be noted that the synthesis of every mole of sucrose from glucose-1-P would form 1 mole of PP_i.

MATERIALS AND METHODS

Source of Materials

Commercially available mung bean seeds and other plant materials used were obtained locally. Duckweed was harvested from a laboratory culture.

All biochemical reagents used were obtained from Sigma Chemical Company, unless noted otherwise. Other chemicals commercially available were of analytical grade.

Special notice is given to the commercial sources of the following materials. Membrane filters (PM30) used in the concentration of protein samples by pressure dialysis were obtained from the Amicon Corporation. Phenol reagent (Folin and Ciocalteu) used in Lowry protein determination was obtained from the Harleco Corporation. DEAE-cellulose and phosphocellulose were obtained from Sigma Chemical Company. Bio-Gel A-1.5m (Agarose beads for gel filtration); AG 1-8X, 200-400 mesh, Cl^- form (anion exchange resin); Bio-Rad protein assay dye reagent; and Bio-Rad protein assay standard II (bovine plasma albumin) were obtained from Bio-Rad Laboratories. Sephadex (G-10 and G-100 medium grade) were obtained from the Pharmacia Fine Chemicals. "Enzyme-grade" ammonium sulfate was obtained from the Mallinckrodt Company. Ferritin was obtained from the Boehringer Mannheim Biochemicals.

Assay Methods for PP_i:F6P 1-Phosphotransferase.

Method A: Forward reaction (standard assay)

Principle. The continuous spectrophotometric assay was based on coupling fructose-1,6-P_2 formation to non-rate-limiting amounts of aldolase, triose-P isomerase, and α-glycerol-P dehydrogenase. With the three coupling enzymes in excess, the rate of fructose-1,6-P_2 formation was equivalent to one-half the rate of NADH oxidation, which was measured by the absorbance decrease at 340 nm.

Reagents.

HEPES-NaOH buffer (pH 7.8), 170 mM, containing 2.1 mM EDTA

Fructose-6-P, 340 mM

Sodium pyrophosphate (Na PP_i), pH 7.8, 17 mM

$MgCl_2$, 102 mM

Fructose-1,6-P_2 aldolase, 40 mU/µl, previously dialyzed against 1 mM EDTA, pH 7.8

Triose-P isomerase, 200 mU/µl, previously dialyzed against 1 mM EDTA, pH 7.8

α-Glycerol-P dehydrogenase, 40 mU/µl, previously dialyzed against 1 mM EDTA, pH 7.8

NADH, 4.1 mM

Activator (17 µM fructose-2,6-P_2)

Procedure. The following were added to a microcuvette with a 1.0-cm light path: 80 µl of HEPES buffer, 5 µl of fructose-6-P, 10 µl of Na PP_i, 10 µl of $MgCl_2$, 5 µl of fructose-1,6-P_2 aldolase, 5 µl of triose-P isomerase, 5 µl of α-glycerol-P dehydrogenase, 10 µl of NADH, 10 µl of fructose-2,6-P_2, a rate-limiting amount of PP_i:F6P 1-phosphotransferase, and water to a volume of 170 µl. The reaction

was initiated by the addition of PP_i:F6P 1-phosphotransferase. A control cuvette minus PP_i measured NADH oxidase and apparent fructose-6-P reductase activities, which was subtracted from the total rate. The rates were measured with a Gilford multiple-sample absorbance-recording spectrophotometer. The cuvette compartment was thermostatted at 30°C. Care was taken to confirm that the rates were constant with time and proportional to the amount of PP_i:F6P 1-phosphotransferase.

Definition of unit and specific activity. One unit was defined as the amount of enzyme that catalyzes the phosphorylation of 1 μmol of fructose-6-P per minute in the standard assay. Specific activity (units per milligram of protein) was based on protein determinations by the method of Whitaker and Granum (134).

Method B: Forward reaction (alternative assay).

Principle. For some determinations, such as measuring the activity with fructose-1,6-P_2 as the activator, a continuous spectrophotometric assay based on coupling P_i formation to non-rate-limiting amounts of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase was used. With the coupling enzymes in excess, the rate of P_i formation was equivalent to the rate of NAD^+ reduction, which was measured by the absorbance increase at 340 nm.

Reagents.

HEPES-NaOH buffer (pH 7.8), 170 mM, containing 2.1 mM EDTA

Fructose-6-P (freed from P_i by chromatography on a column of Sephadex G-10), 170 mM

Sodium pyrophosphate (Na PP_i), pH 7.8, 17 mM

$MgCl_2$, 102 mM

DL-glyceraldehyde-3-P (prepared from DL-glyceraldehyde-3-P diethylacetal; Sigma Chemical Co.), 17 mM of the D-enantiomorph

ADP (vanadium free), 17 mM

Glyceraldehyde-3-phosphate dehydrogenase, 40 mU/µl, previously dialyzed against 1 mM EDTA, pH 7.8

3-Phosphoglycerate kinase, 80 mU/µl, previously dialyzed against 1 mM EDTA, pH 7.8

NAD^+, 8.2 mM

Activator (3.4 mM fructose-1,6-P_2)

Procedure. The following were added to a microcuvette with a 1.0-cm light path: 80 µl of HEPES buffer, 10 µl of fructose-6-P, 10 µl of Na PP_i, 10 µl of $MgCl_2$, 5 µl of DL-glyceraldehyde-3-P, 10 µl of ADP, 5 µl of glyceraldehyde-3-P dehydrogenase, 5 µl of 3-phosphoglycerate kinase, 10 µl of NAD^+, 5 µl of fructose-1,6-P_2, a rate-limiting amount of PP_i:F6P 1-phosphotransferase, and water to a volume of 170 µl. The reaction was initiated by the addition of PP_i:F6P 1-phosphotransferase. A control cuvette minus the enzyme preparation measures P_i release due to non-enzymatic hydrolysis of glyceraldehyde 3-phosphate, which was subtracted from the total rate. This assay is valid only when used with phosphatase-free preparations. In addition, the fructose-6-phosphate reagent must be essentially free of P_i so that the initial absorbance values can be kept low. The rates were measured with a Gilford multiple-sample absorbance-recording spectrophotometer. The cuvette compartment was thermostatted at 30°C.

Method C: Reverse reaction.

Principle. This continuous spectrophotometric assay was based on coupling fructose-6-P formation to non-rate-limiting amounts of phosphoglucose isomerase and glucose-6-P dehydrogenase. With the coupling enzymes in excess, the rate of fructose-6-P formation was equivalent to the rate of $NADP^+$ reduction, which was measured by the absorbance increase at 340 nm.

Reagents.

HEPES-NaOH buffer (pH 7.8), 170 mM, containing 2.1 mM EDTA

Fructose-1,6-P_2, 17 mM

Sodium phosphate (Na P_i), pH 7.8, 17 mM

$MgCl_2$, 102 mM

Phosphoglucose isomerase, 80 mU/µl, previously dialyzed against 1 mM EDTA, pH 7.8

Glucose-6-P dehydrogenase, 40 mU/µl, previously dialyzed against 1 mM EDTA, pH 7.8

$NADP^+$, 8.2 mM

Procedure. The following were added to a microcuvette with a 1.0-cm light path: 80 µl of HEPES beffer, 10 µl of fructose-1,6-P_2, 10 µl of Na P_i, 10 µl of $MgCl_2$, 5 µl of phosphoglucose isomerase, 5 µl of glucose-6-P dehydrogenase, 10 µl of $NADP^+$, a rate-limiting amount of PP_i:F6P 1-phosphotransferase, and water to a volume of 170 µl. Fructose-1,6-P_2 served both as the substrate and activator. The reaction was initiated by the addition of PP_i:F6P 1-phosphotransferase. A control cuvette minus Na P_i measured fructose-1,6-bisphosphatase activity, which was subtracted from the total rate. The rates were measured with a Gilford multiple-sample absorbance-recording

spectrophotometer. The cuvette compartment was thermostatted at 30°C.

Other Enzyme Assays.

All of the following assays in which pyridine nucleotides were monitored were done at 30°C and 340 nm with a Gilford automated recording spectrophotometer.

ATP-dependent phosphofructokinase. The standard assay was a continuous enzyme-coupled assay wherein the oxidation of NADH was followed. The reaction mixture (170 µl) consisted of 80 mM HEPES-NaOH (pH 7.4), 10 mM fructose-6-P, 1.0 mM ATP, 6 mM $MgCl_2$, 0.24 mM NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, and rate-limiting amounts of the enzyme.

Inorganic pyrophosphatase. Catalytic hydrolysis of PP_i was measured by the appearance of P_i using Josse's (135) modified Fiske and SubbaRow method. The incubation mixture (0.3 ml) contained 20 µmol of 2-amino-2-methyl-1,3-propanediol-chloride (pH 9.1), 0.4 µmol of $MgCl_2$, 0.2 µmol of $Na_4P_2O_7$, and enzyme preparation. The reaction was incubated for 20 min at 30°C, stopped by chilling to 0°C, followed by addition of 0.7 ml of a mixture containing 0.1 ml of 5N H_2SO_4, 0.1 ml of 2.5% ammonium molybdate (tetrahydrate), 0.1 ml of 3% $NaHSO_3$ - 1% p-methylaminophenol sulfate (Elon), and 0.4 ml of water. After 10 min at 25°C, the absorbance at 660 nm was determined.

Fructose-1,6-bisphosphatase. The enzyme was assayed by a phosphoglucose isomerase-glucose 6-phosphate dehydrogenase-linked assay, wherein the reduction of $NADP^+$ was followed. The reaction mixture (170 µl) consisted of 80 mM HEPES-NaOH (pH 7.4), 2.0 mM

fructose-1,6-P_2, 6 mM $MgCl_2$, 0.24 mM $NADP^+$, 0.2 unit of phosphoglucose isomerase, 0.2 unit of glucose-6-phosphate dehydrogenase and rate-limiting amounts of the enzyme.

Phosphoglucose isomerase. The enzyme was assayed by a glucose-6 phosphate dehydrogenase-linked assay, wherein the reduction of $NADP^+$ was followed. The reaction mixture (170 µl) consisted of 80 mM HEPES-NaOH (pH 7.4), 10 mM fructose-6-P, 6 mM $MgCl_2$, 0.24 mM $NADP^+$, 0.2 unit of glucose-6-phosphate dehydrogenase, and a rate-limiting amount of the enzyme.

Adenylate kinase. This enzyme was assayed by a pyruvate kinase-lactate dehydrogenase-linked assay wherein the oxidation of NADH was followed. The reaction mixture (170 µl) consisted of 80 mM HEPES-NaOH (pH 7.4), 1.0 mM AMP, 0.5 mM ATP, 3 mM phosphoenolpyruvate, 6 mM $MgCl_2$, 0.24 mM NADH, non-rate-limiting amounts of the coupling enzymes, and rate-limiting amounts of adenylate kinase.

Assays for Molecular Weight Standards.

All of the following assays in which the oxidation of NADH was monitored were done at 30°C and 340 nm with a Gilford automated recording spectrophotometer.

Pyruvate kinase. Rabbit muscle pyruvate kinase was determined by measuring the production of pyruvate from phosphoenolpyruvate by a lactate dehydrogenase-linked assay. The reaction mixture (170 µl) contained 80 mM HEPES-NaOH (pH 7.8), 6 mM $MgCl_2$, 1 mM ADP, 0.5 mM phosphoenolpyruvate, 0.24 mM NADH, non-rate-limiting amounts of lactate dehydrogenase, and 10 µl of the column fractions.

Aldolase. Rabbit muscle aldolase was assayed by a triose-P isomerase-α-glycerol-P dehydrogenase-linked assay. The reaction mixture (170 µl) contained 80 mM HEPES-NaOH (pH 7.8), 6 mM $MgCl_2$, 1 mM fructose-1,6-P_2, 0.24 mM NADH, 1.0 unit of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, and 10 µl of the column fractions.

Hexokinase. Yeast hexokinase was assayed by a pyruvate kinase--lactate dehydrogenase-linked assay. The reaction mixture (170 µl) contained 80 mM HEPES-NaOH (pH 7.8), 6 mM $MgCl_2$, 5 mM glucose, 0.5 mM ATP, 0.24 mM NADH, 2 mM phosphoenolpyruvate, 1 unit of pyruvate kinase, 1 unit of lactate dehydrogenase, and 10 µl of the column fractions.

Ferritin. Ferritin was determined by reading the absorbance at 280 nm (room temperature) of 50-µl column fractions diluted to 250 µl with the buffer used to elute the column.

Protein Determination.

Protein was determined by the procedure of Lowry et al. (136), by the Bradford method (137) using bovine serum albumin as the standard, or by the method of Whitaker and Granum (134), which does not need a standard protein.

Pressure Dialysis Concentration of Protein.

Pressure dialysis was performed using an Amicon Diaflo apparatus (400-ml and 50-ml capacity), equipped with a PM30 membrane filter, having a 30,000-molecular-weight range cutoff. Concentration of the protein was performed under nitrogen pressure (30 psi). When not in use, the filter was stored at 4°C in 20% ethyl alcohol after first washing it with distilled water.

Conductivity Measurements.

Column fractions from purification steps were assayed for salt concentration using a standard conductivity meter with variable-conductance control. Samples of 0.05-ml volume were diluted 100-fold with de-ionized water and read against a diluted buffer blank. The instrument was calibrated using the highest and lowest salt concentrations of the gradient in the appropriate buffer.

Preparation of Fructose-2,6-Bisphosphate.

Fructose-2,6-P_2 was chemically synthesized as described by Van Schaftingen and Hers (27). The procedure involved (i) intramolecular cyclization of fructose-1,6-P_2 to fructose 1,2 cyclic,6-bisphosphate, (ii) ring opening by alkaline treatment, (iii) neutralization and treatment with fructose-1,6-bisphosphatase, and (iv) separation by chromatography through Bio-Rad AG 1-8X with elution by a gradient of NaCl. Fructose-2,6-P_2 was quantified by measuring the fructose-6-P and P_i revealed upon acid hydrolysis. The fructose-6-P concentration was determined by enzymatic end-point assay using phosphoglucose isomerase and glucose-6-P dehydrogenase. The P_i concentration was determined by enzymatic end-point assay using glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase. The separation of fructose-2,6-P_2 from P_i and fructose-6-P through Bio-Rad AG 1-8X is shown in Figure 2.

Figure 2. Purification of chemically synthesized fructose-2, 6-P_2 by chromatography on a column of Bio-Rad AG 1-X8. The fructose-2, 6-P_2 corresponds to the fractions that gave equal concentrations of acid-revealed fructose-6-P and P_i (fractions 56-68). The fructose-6-P concentration was determined by enzymatic end-point assay by coupling to phosphoglucose isomerase and glucose-6-P dehydrogenase. The P_i concentration was determined by enzymatic end-point assay by coupling to glyceraldehyde-3-P dehydrogenase and 3-phosphoglycerate kinase.

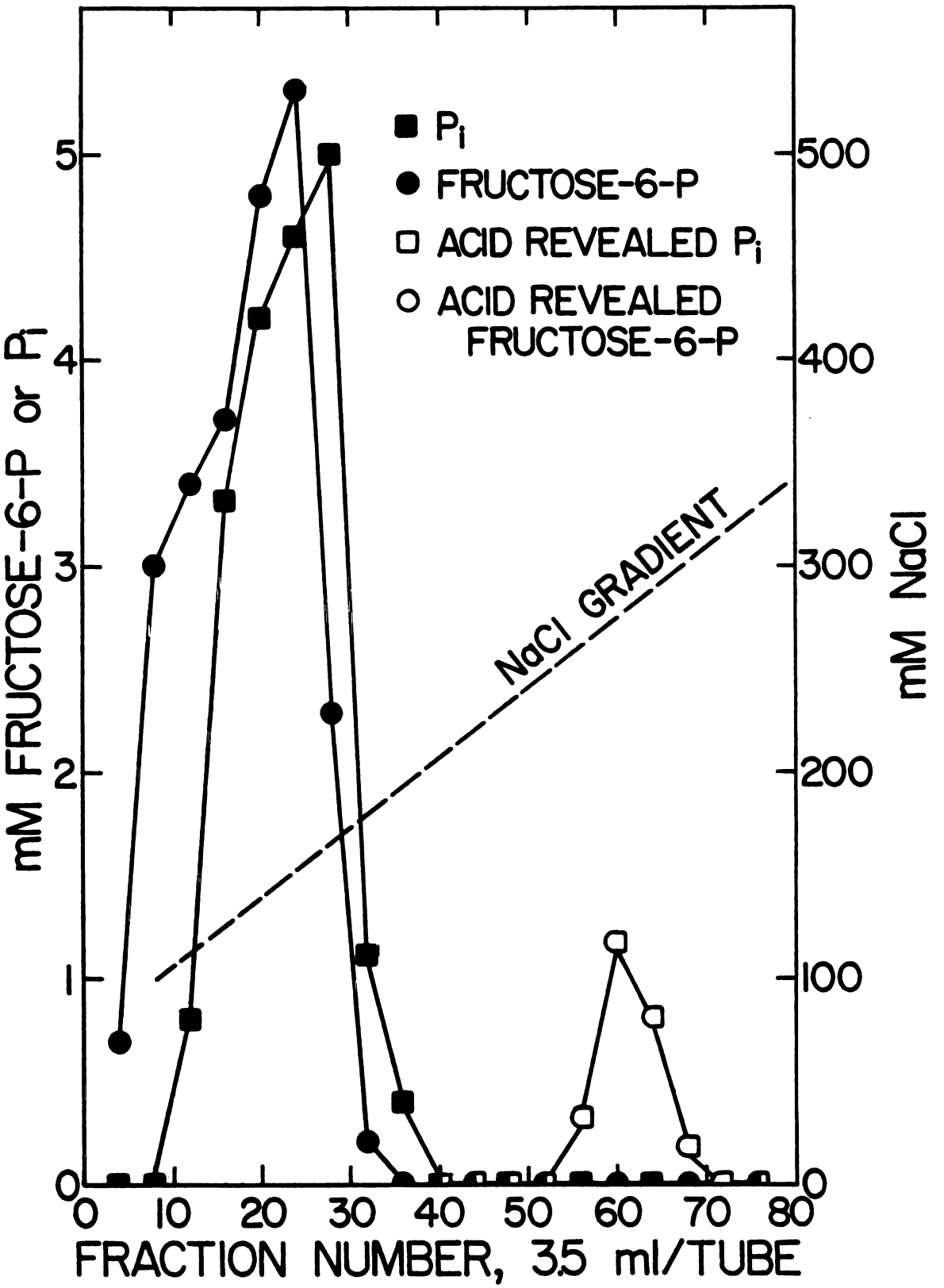
Pi
FRUCTOSE-6-P
ACID REVEALED Pi
ACID REVEALED FRUCTOSE-6-P
NaCl GRADIENT
mM FRUCTOSE-6-P or Pi
mM NaCl
FRACTION NUMBER, 3.5 ml/TUBE
0
1
2
3
4
5
100
200
300
400
500
0
10
20
30
40
50
60
70
80

Figure 2

Preparation of Fructose-6-P Free of P_i by Chromatography Through Sephadex G-10.

One-half gram of fructose-6-P was dissolved in water to 1.7 ml and loaded onto a Sephadex G-10 column (2.6 x 100 cm) previously equilibrated with double-distilled H_2O. The sugar phosphate was eluted with double-distilled H_2O at room temperature at a linear flow rate of 2.5 cm/hr. Two-hundred-twenty 1-ml column fractions were collected. Fructose-6-P was determined by a phosphoglucose isomerase-glucose-6-P dehydrogenase-linked end-point assay. The reaction mixture (170 µl) contained 80 mM HEPES-NaOH (pH 7.8), 6 mM $MgCl_2$, 0.5 mM $NADP^+$, 2 units of phosphoglucose isomerase, 1 unit of glucose-6-P dehydrogenase and 2 µl of 1/20 dilution of the column fractions. The absorbance change after the completion of the reaction was read at 340 nm. The concentration of fructose-6-P was calculated from the equivalent NADH formed. The P_i was determined by a glyceraldehyde-3-P dehydrogenase-3-phosphoglycerate kinase-linked end-point assay. The reaction mixture (170 µl) contained 80 mM HEPES-NaOH (pH 7.8), 6 mM $MgCl_2$, 1 mM ADP (vanadium free), 0.5 mM of the D-enantiomorph of DL-glyceraldehyde-3-P (prepared from DL-glyceraldehyde 3-phosphate diethylacetal), 0.5 mM NAD^+, 1 unit of glyceraldehyde 3-phosphate dehydrogenase, 2 units of 3-phosphoglycerate kinase, and 15 µl of the column fraction. The absorbance change after the completion of the reaction was read at 340 nm. The concentration of P_i was calculated from the equivalent NADH formed.

Partial Purification and Elucidation of Some Properties of PP_i:F6P 1-phosphotransferase.

A preliminary purification scheme devised earlier in this investigation has been described in the article of Sabularse and Anderson (138). This enzyme preparation (100-fold pure), along with three assay procedures, was utilized to elucidate the absolute requirement for fructose-6-P and PP_i as well as for a divalent metal ion (Mg^{++}), activation by a product of the reaction (fructose-1,6-P_2), activation by glucose-1,6-P_2, and the effect of some compounds on the activity.

Further Purification and Elucidation of Additional Properties of PP_i:F6P 1-phosphotransferase.

Another preliminary purification scheme is described by Sabularse and Anderson (139) and elaborated on in an article by Anderson and Sabularse (140). This enzyme preparation (670-fold pure) was used in the determination of the K_A for fructose-1,6-P_2, glucose-1,6-P_2 and fructose-2,6-P_2; the K_m for fructose-6-P and the relative V_{max}, in the absence and in the presence of a hexose bisphosphate activator; and as a reagent in the demonstration of the natural occurrence of fructose-2,6-P_2 in mung beans.

Final Purification and Partial Separation of Two Forms of PP_i:F6P 1-Phosphotransferase and Characterization of Their Properties.

Preparation of germinated seeds. Commercially available mung bean seeds (500 g) were soaked in distilled water for 12 hrs in the dark at 30°C. The soaked seeds were spread between 8 layers of moist cheese

cloth and allowed to germinate for an additional 12 hrs in the dark at 30°C.

General. All purification steps were conducted at 0-4°C. The assay employed was Method A (described earlier) with 1 μM fructose-2,6-P_2 as the activator.

Preparation of crude extract. The germinated seeds were separated from the seed coat by repeated washing and hand-picking. The germinated beans, free of the seed coats, were suspended 1:1 (v/v) in buffer A (50 mM Tris-acetate, pH 7.0, containing 1 mM EDTA and 180 mM Na acetate) and ground with a mortar and pestle. The homogenate was filter-squeezed through 8 layers of cheese cloth and clarified by centrifugation at 13,000 x g for 20 min. The supernatant solution was designated as the crude extract.

Ammonium sulfate precipitate. To 2030 ml of the crude extract, 369 g of ammonium sulfate was added slowly to bring the concentration to 30% saturation. The solution was stirred for 30 min and then centrifuged at 13,000 x g for 20 min. The 2170 ml of supernatant was decanted and 253 g more of ammonium sulfate was added to bring the concentration to 50% saturation. After 30 min., the solution was centrifuged once again. The supernatant was discarded and the pelleted material was suspended in buffer B (10 mM Tris-acetate, pH 7.3 containing 0.1 mM EDTA and 10% glycerol) to 146 ml.

DEAE-cellulose chromatography. The pooled 30-50% ammonium sulfate fraction was dialyzed against three 3-liter changes of buffer B and loaded onto a DEAE-cellulose column (4.7 x 26 cm) pre-equilibrated with buffer B. The column was washed with 5 bed-volumes of buffer B before the protein was eluted with 4.5-liter linear gradient of 0 to 0.4 M KCl

in the same buffer. Fractions 62 to 95, containing most of the activity, were pooled for further purification.

Phosphocellulose chromatography I. The pooled fractions from the DEAE-cellulose column were concentrated to 77 ml by pressure fltration through a PM30 membrane. The concentrated pooled fractions were dialyzed against 3 changes of 2 liters each of buffer C (5 mM Na-PIPES, pH 6.6 containing 10 mM KCl, 0.1 mM Na EDTA and 10% glycerol), and loaded onto a phosphocellulose column (4.0 x 14.5 cm) previously equilibrated with buffer C. The loading was followed by passing through the column about 5 bed-volumes of buffer C before the enzyme was eluted with buffer C containing 34 mM Na PP_i with the pH maintained at 6.6. Fractions 50-115, containing most of the activity, were pooled for further purification.

Phosphocellulose chromatography II. The pooled fractions from phosphocellulose chromatography I were concentrated to 77 ml by pressure filtration through a PM30 membrane. The concentrated pooled fractions were dialyzed against three 2-liter changes of buffer C, and loaded onto a second phosphocellulose column (2.6 x 11.0 cm) previously equilibrated with buffer C. The loading was followed by passing through the column about 4 bed-volumes of buffer C before the enzyme was eluted by a 1500-ml linear gradient of 0 to 0.5 M KCl in buffer C. Fractions 69-92, containing most of the activity, were pooled for further purification.

Bio-Gel A-1.5m. The pooled fractions from phosphocellulose chromatography II were concentrated to 10 ml by pressure dialysis through a PM30 membrane. The concentrated pooled fractions were applied to a Bio-Gel A-1.5m column (3.4 x 95 cm) equilibrated with

buffer B with the addition of 20 mM KCl, and then eluted with the same buffer. Fractions (5-ml each) were collected and those with high specific activity for the enzyme (fractions 82 through 123) were combined.

Separation of two forms of the enzyme. PP_i:F6P 1-Phosphotransferase in the above fraction was separated into two forms by further chromatography on Bio-Gel A-1.5m as detailed in the Results section.

Partial Purification and Determination of Some Properties of ATP-dependent Phosphofructokinase.

ATP-dependent phosphofructokinase was extracted and partially purified from 50 g of mung beans germinated in the dark at 30°C for 24 hrs. The sprouts were separated from their seed coats, suspended 1:1 (v/v) in buffer (50 mM Tris-acetate, pH 7.0, containing 5 mM dithiothreitol, 1 mM EDTA, and 180 mM K acetate, and were ground with a mortar and pestle. The homogenate was filter-squeezed through 8 layers of cheese cloth and clarified by centrifugation at 22,000 x g for 20 min. The ATP-dependent phosphofructokinase in the supernatant fluid (the crude extract) was partially purified (20 fold) by the following procedure: precipitation with ammonium sulfate (30-45% saturation); chromatography on Sephadex G-100 (the ammonium sulfate precipitate was dissolved to 10 ml with the elution buffer and loaded onto a 500-ml bed-volume column, and was then eluted with 10 mM Tris-acetate buffer at pH 7.0 containing 1 mM DTT, 0.1 mM EDTA and 180 mM K acetate); chromatography on DEAE-cellulose I (the active fractions from Sephadex G-100 column chromatography step were pooled, dialyzed, and loaded onto a 20-ml bed-volume column previously equilibrated with 10 mM Na-MES, pH

6.2, containing 1 mM DTT and 0.1 mM EDTA, and then the protein was eluted by 20 ml each of a step gradient of 0.1, 0.2, 0.3 and 0.4 M KCl in the pH 6.2 Na-MES buffer); and chromatography on DEAE-cellulose II (0.2 and 0.3 M KCl eluates from the DEAE-cellulose I step were pooled, dialyzed, and loaded onto a second 20-ml bed-volume column previously equilibrated with the pH 6.2 Na-MES buffer, and the protein was eluted by a 200-ml linear gradient of 0-400 mM KCl in the pH 6.2 Na-MES buffer). The tubes containing most of the phosphofructokinase activity devoid of PP_i:F6P 1-phosphotransferase were combined, concentrated by pressure filtration through a PM30 membrane, and stored frozen. This enzyme preparation was designated as DEAE-cellulose II ATP-dependent phosphofructokinase.

The DEAE-cellulose II ATP-dependent phosphofructokinase preparation was used in the determination of the saturation curves for fructose-6-P and ATP, and the effect of pH and some metabolites on the activity.

Extraction of the Natural Activator (Fructose-2,6-P_2) from Mung Beans.

The natural activator was extracted from germinated mung bean (Phaseolus aureus Roxb.) seeds. Twenty-five grams of mung bean seeds, purchased locally, were germinated in the dark at 30°C for 24 hr. The sprouts were separated from their seed coats, suspended 1:1 (v/v) in buffer (100 mM glycine-NaOH, pH 10.4, containing 300 mM NaCl) and ground with a mortar and pestle. The homogenate was squeeze-filtered through 8 layers of cheese cloth and the filtrate was adjusted to pH 9.4 with NaOH, then clarified by centrifugation at 22,000 x g for 20

min. The crude extract was pressure filtered through a PM30 ultrafiltration membrane and the filtrate was diluted with water six-fold before being introduced to a column (1 x 15 cm) of Bio-Rad AG 1-X8 anion exchange resin (Cl^- form, 200 to 400 mesh). The column was washed with 10 bed-volumes of water and the sugar phosphate was eluted with a linear gradient (100 ml, 30 ml/h) of 100 to 400 mM NaCl. Eighty-four 1.2-ml fractions were collected. Fructose-1,6-P_2 was determined by enzymatic end-point assay using fructose-1,6-P_2 aldolase, triose-P isomerase and α-glycerol-P dehydrogenase. The unknown activator was located and measured by its ability to activate PP_i:F6P 1-phosphotransferase; if the column fraction also contained fructose-1,6-P_2, PP_i:F6P 1-phosphotransferase was added to the assay mix after the initial rapid decrease in absorbance.

A scaled-up isolation procedure starting with 1000 g of germinated beans was conducted in which fructose-1,6-bisphosphatase treatment and a second anion-exchange chromatography step were used to separate the activator from fructose-1,6-P_2. The fractions containing the activator were pooled and concentrated by lyophilization. A portion of the concentrate was subjected to mild acid hydrolysis (pH 2.5, room temperature, for 30 min) and the resulting hydrolysate was neutralized with NaOH, and examined for fructose-6-P by specific enzyme-coupled assays employing (i) glucose 6-phosphate isomerase (EC 5.3.1.9) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and (ii) phosphofructokinase (EC 2.7.1.11), fructose-1,6-P_2 aldolase, triose-P isomerase, and α-glycerol-P dehydrogenase.

Survey of PP_i:F6P 1-Phosphotransferase and Fructose 2,6-Bisphosphate in Other Plant Species.

Assay Method A with fructose-2,6-P_2 as activator was employed to determine the presence of the enzyme in several plant species. In each plant species that was surveyed for the enzyme, crude extract from about 25 g of sample was prepared in similar manner as in the preparation of crude extract from sprouted mung bean. The crude extracts were assayed for the enzyme both in the presence and absence of fructose-2, 6-P_2. The blank was either no PP_i or fructose-6-P. Protein was determined by the method of Whitaker and Granum (134).

Using the purified PP_i:F6P 1-phosphotransferase as a reagent and employing assay Method A, a survey of the occurrence of fructose-2,6-P_2 in some plant species was conducted. A five to twenty-five gram sample of each plant species was used and the extraction and isolation procedures were as described earlier for the extraction and isolation of the natural activator from sprouted mung beans. The amount of fructose-2,6-P_2 per unit weight of sample was estimated by noting the relative area of the elution profile from the AG 1-X8 anion exchange column and relating it to the weight of plant material that was used during the extraction.

RESULTS

Detection of PP_i:F6P 1-Phosphotransferase in an Extract From Germinated Mung Beans.

With minor modifications of published assay methods (17, 18), this enzyme activity was detected in the crude extract [prepared by following the procedure of Nomura and Akazawa (141) for the preparation of crude extract for enzymatic assay for sucrose-P synthase from germinated rice seeds] of sprouted mung beans. However, the apparent specific activity was very much lower than that of the ATP dependent phosphofructokinase, being 1/40 even at the 24-hour germination time, when both the enzymes were at their highest apparent specific activities (Figure 3). In contrast, in other species where the enzyme has been reported (17-21), the PP_i:F6P 1-phosphotransferase activity is much greater than that of the ATP-dependent phosphofructokinase. Because the existence of this enzyme in C_3 plants had not been previously recognized, the research was pursued on the basis of the idea that the apparent low specific activity in mung beans was due to suboptimal assay conditions or the existence of the enzyme in an inhibited or unactivated state.

Figure 3. Apparent specific activities of PP_i:F6P 1-Phosphotransferase and ATP-dependent phosphofructokinase at different times of germination prior to the discovery of activation by hexose bisphosphates. The assay mixture (170 µl) for PP_i:F6P 1-Phosphotransferase contained 80 mM HEPES-NaOH buffer (pH 7.4), 25 mM Na fructose-6-P, 1.0 mM PP_i, 3.0 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of glyceraldehyde-3-P dehydrogenase, and 10 µl of crude extract. A similar assay mixture was employed for ATP-dependent phosphofructokinase except that 1 mM ATP replaced PP_i, and only 10 mM Na fructose-6-P and 5 µl of crude extract were used. The observed velocities were divided by 2 to correct for the 2-fold amplfication. Protein was determined by the method of Lowry et al. 1-PT = 1-phosphotransferase. PFK = ATP-dependent phosphofructokinase.

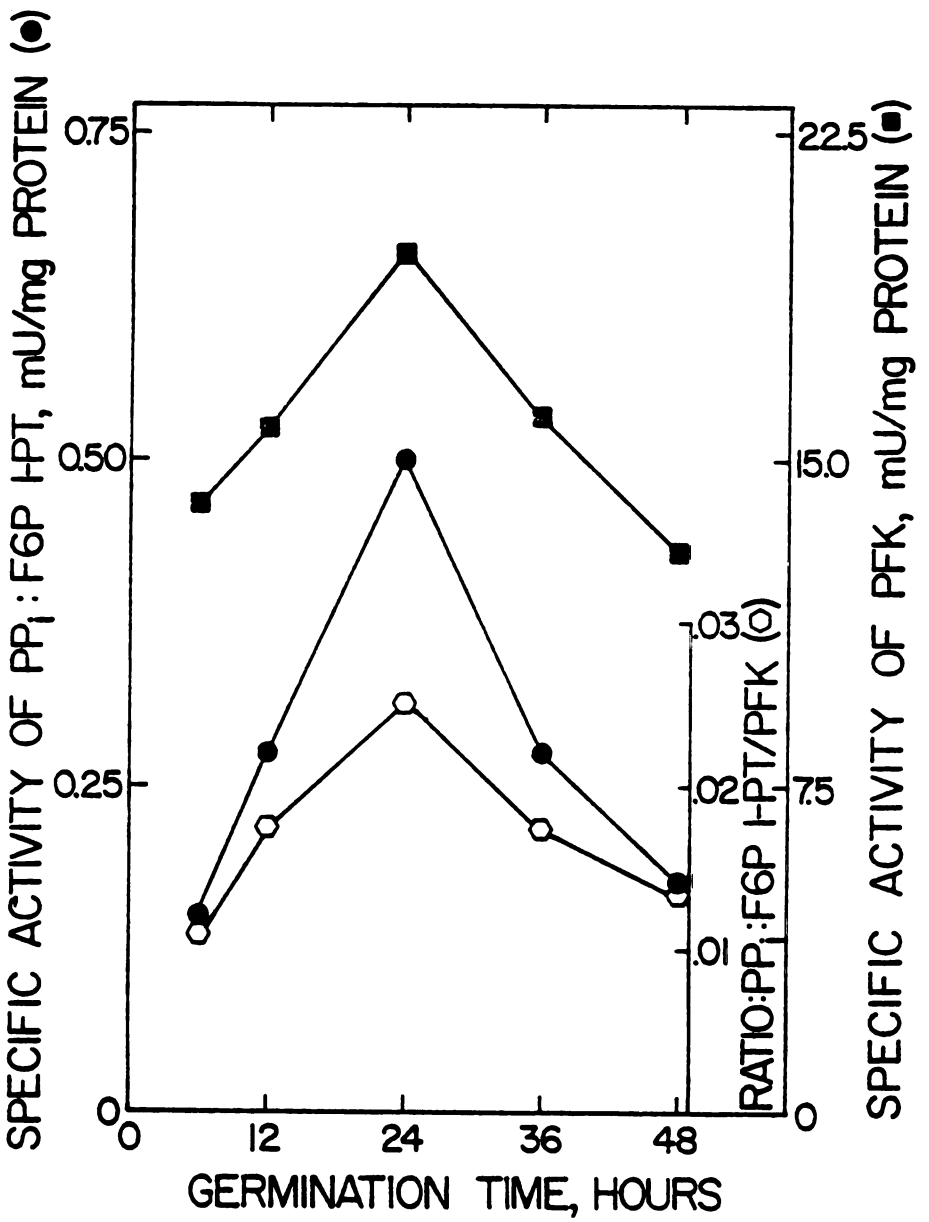
SPECIFIC ACTIVITY OF PPi : F6P I-PT, mU/mg PROTEIN (●)
0.75
0.50
0.25
0
0
12
24
36
48
GERMINATION TIME, HOURS
RATIO:PPi:F6P I-PT/PFK (○)
.03
.02
.01
22.5
15.0
7.5
0
SPECIFIC ACTIVITY OF PFK, mU/mg PROTEIN (■)

Figure 3

Partial Purification and Some Properties of PP_i:F6P 1-Phosphotransferase.

For use in a preliminary investigation, the enzyme was partially purified (100 fold) as described by Sabularse and Anderson (138). The purification steps consisted of ammonium sulfate precipitation, DEAE cellulose chromatography, Bio-Gel A-1.5m chromatography and phosphocellulose chromatography. The enzyme preparation was essentially free of enzymes (e.g., ATP-dependent phosphofructokinase, adenylate kinase, phosphoglucose isomerase and fructose-1,6-bisphosphatase) that could interfere with the assay.

Requirement for specific substrates and a divalent metal ion. The partially purified enzyme had an absolute requirement for both fructose-6-P and PP_i. ATP could not substitute for PP_i (Table 1, Assay I). Other compounds (1 mM) that could not replace PP_i as the phosphoryl donor included UTP, ADP, UDP, P_i, AMP and phosphoenolpyruvate. There was also an absolute requirement for a divalent metal ion such as Mg^{++}. Mn^{++} and Co^{++} could replace Mg^{++} but were less effective. Zn^{++}, Cu^{++}, Fe^{++} and Ca^{++} did not substitute for Mg^{++} (Table 2). Several compounds that were tested as possible effectors for the enzyme had little or no apparent effect with the assay conditions used (Table 3); however, the observed inhibition by P_i was significant as will be documented later in this thesis.

Activation of PP_i:F6P 1-phosphotransferase by a product of the reaction. Several observations on the kinetics of the assay suggested that the enzyme was activated by a product of the reaction, fructose-1,6-P_2: (i) whereas omitting triose-P isomerase would be expected to halve the rate of NADH oxidation in an aldolase-triose-P

Footnotes to Table 1.

[a]The assay mix (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 20 mM Na fructose-6-P, 1.0 mM Na PP_i, 6.0 mM $MgCl_2$, 0.3 mM Na NADH, 100 mU of fructose-1,6-P_2 aldolase, 2.0 U of triose-P isomerase, 200 mU of α-glycerol-P dehydrogenase, and partially purified PP_i:F6P 1-phosphotransferase. The observed velocity was divided by 2 to correct for the 2-fold amplification.

[b]The assay mix (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 20 mM Na fructose-6-P, 1.0 mM Na PP_i, 6.0 mM $MgCl_2$, 0.3 mM Na NAD^+, 1.0 mM glyceraldehyde-3-P, 3.0 mM Na ADP, 100 mU of D-glyceraldehyde-3-P dehydrogenase, 200 mU of 3-P-glycerate kinase, and partially purified PP_i:F6P 1-phosphotransferase. The reported velocities have been corrected for a background rate due to glyceraldehyde-3-P hydrolysis (see Figure 7, Curve 3).

[c]The assay mix (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 2.0 mM Na fructose-1,6-P_2, 2.0 mM Na P_i, 6.0 mM $MgCl_2$, 0.3 mM Na $NADP^+$, 100 mU of phosphohexose isomerase, 200 mU of D-glucose-6-P dehydrogenase, and partially purified PP_i:F6P 1-phosphotransferase.

[d]All assays contained 1.7 µg of the PP_i:F6P 1-phosphotransferase preparation. The rates observed for the three assays (i.e., 2.8, 3.2, and 0.92) are not identical because of the influence of differing concentrations of contaminating and component ions on the reaction velocity.

[e]The maximal slope was achieved after the optimally activating concentration of fructose-1,6-P_2 was formed in the reaction.

Table 1. Some properties of PP_i:F6P 1-phosphotransferase partially purified from mung bean sprouts, as determined by three different assay methods.

Assay	Conditions: Omissions	Conditions: Additions	Reaction Velocity[d] (nmol/min)
I[a] (measures fructose-1,6-P_2 formed)	None	None	0.14
	PP_i:F6P 1-phospho-transferase	None	0.00
	Fructose-6-P	None	0.00
	$MgCl_2$	None	0.00
	PP_i	None	0.00
	PP_i	ATP (1.0 mM)	0.00
	None	Glucose-1,6-P_2 (0.3 mM)	1.3
	None	Glucose-1,6-P_2 (1.5 mM)	2.8
II[b] (measures P_i formed)	None	None	3.2 (max slope)[e]
	PP_i:F6P 1-phospho-transferase	None	0.00
	Fructose-6-P	None	0.00
	PP_i	None	0.00
	None	Fructose-1,6-P_2 (0.3 mM)	3.2
	None	Glucose-1,6-P_2	3.2
III[c] (measures reverse reaction)	None	None	0.92
	PP_i:F6P 1-phospho-transferase	None	0.00
	Fructose-1,6-P_2	None	0.00
	P_i	None	0.00
	None	Glucose-1,6-P_2 (0.3 mM)	0.92

Table 2. Effect of various divalent metals on PP_i:F6P 1-phosphotransferase. The assay mix (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 20 mM Na fructose-6-P, 1.0 mM $Na_4P_2O_7$, 6.0 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 1.5 mM glucose-1,6-P_2, 10 µl of partially purified PP_i:F6P 1-phosphotransferase and the indicated divalent metal.

Divalent metal	Concentration	Activity (mU)
None	-	0.04
None + 1 mM EDTA	-	0.00
Mg^{++}	2 mM	1.20
Co^{++}	2 mM	0.76
Mn^{++}	2 mM	0.34
Ca^{++}	2 mM	0.04
Fe^{++}	2 mM	0.04
Cu^{++}	2 mM	0.04
Zn^{++}	2 mM	0.04

Table 3. Effect of some compounds on PP_i:F6P 1-phosphotransferase. The assay mix (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 20 mM Na fructose-6-P, 1.0 mM $Na_4P_2O_7$, 6.0 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1-6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 10 µl of partially purified PP_i:F6P 1-phosphotransferase and the indicated amount of the possible effector tested. The putative effector was preincubated with the reaction mixture for 10 min before the reaction initiated by the addition of fructose-6-P.

Concentration of effector	Activity (nmol fructose-1,6-P_2 formed/min)		Effect
	No effector	With effector	
1 mM UDP-Glucose	0.60	0.67	12% Activation
1 mM Glucose-1-P	"	0.66	10% "
1 mM P_i	"	0.45	25% Inhibition
1 mM ATP	"	0.50	17% "
1 mM UDP	"	0.53	11% "
1 mM ADP	"	0.60	no effect
1 mM UTP	"	"	"
1 mM Phosphoenolpyruvate	"	"	"
1 mM AMP	"	"	"
1 mM Citrate	"	"	"
1 mM Glucose-6-P	"	"	"
1 mM 3-Phospho-glyceric acid	"	"	"
1 mM 1,3-Diphospho-glyceric acid	"	"	"
0.08 mM NAD^+	"	"	"

isomerase-α-glycerol-P dehydrogenase-linked assay, the omission actually caused a time-dependent increase (Figure 4, Curves 1 and 2); (ii) the time required to achieve the apparent activation of PP_i:F6P 1-phosphotransferase in the absence of triose-P isomerase was decreased by the addition of glyceraldehyde-3-P (Figure 4, Curve 3); (iii) increasing the concentration of PP_i:F6P 1-phosphotransferase resulted in more than a proportionate increase in the reaction velocity (Figure 5, Curve 1), but when triose-P isomerase was omitted from the assay, the rate was essentially proportional to the PP_i:F6P 1-phosphotransferase concentration (Figure 5, Curve 2) and (iv) delaying the addition of aldolase to the assay mixture for 5 min resulted in an absorbance decrease that was considerably greater than that in a control in which aldolase was added at the start of the reaction (data not shown). It was deduced from these observations that varying the conditions influenced the steady-state concentration of fructose-1,6-P_2, and that when the concentration was elevated, PP_i:F6P 1-phosphotransferase was activated. Thus, omitting triose-P isomerase caused glyceraldehyde-3-P to accumulate, thereby increasing the fructose-1,6-P_2 concentration by mass action; the addition of exogenous glyceraldehyde-3-P in the absence of triose-P isomerase potentiated this effect; increasing the PP_i:F6P 1-phosphotransferase concentration at constant levels of coupling enzymes increased the steady-state concentration of fructose-1,6-P_2 even in the presence of triose-P isomerase, thus accounting for the apparent enzyme-concentration-dependent activation of PP_i:F6P 1-phosphotransferase; and delaying the addition of aldolase resulted in a temporary accumulation of fructose-1,6-P_2, thereby activating the PP_i:F6P 1-phosphotransferase.

Figure 4. Apparent time-dependent activation of PP_i:F6P 1-phosphotransferase in the absence of triose-P isomerase and apparent stimulation of activity by glyceraldehyde-3-P. Curve 1, complete reaction mixture (Assay I in Table 1, uncorrected for 2-fold amplification); Curve 2, complete minus triose-P isomerase; Curve 3, complete minus triose-P isomerase and plus 0.16 mM glyceraldehyde-3-P.

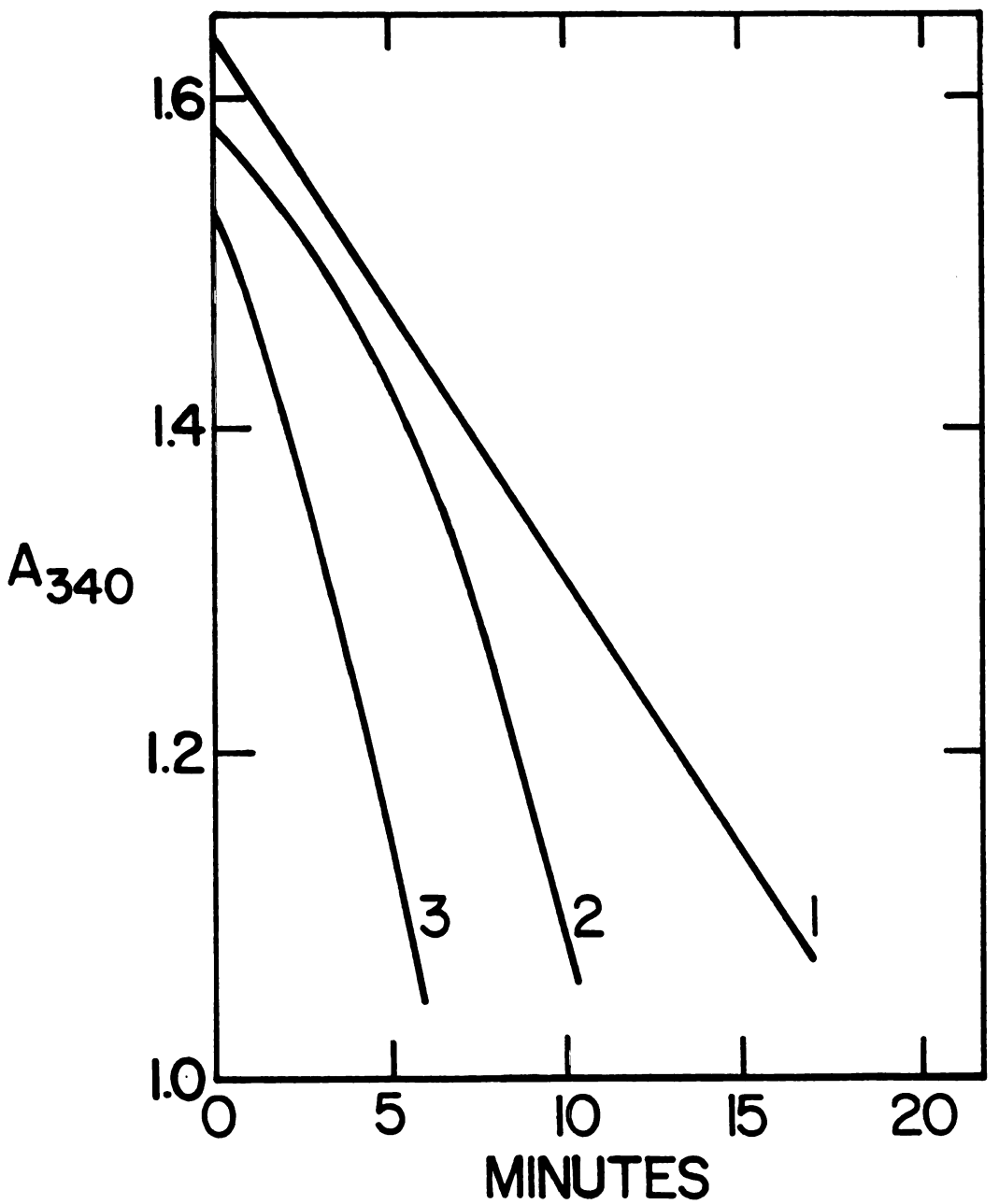

Figure 4

Figure 5. Proportionality of PP_i:F6P 1-phosphotransferase acitivity and enzyme concentration. Curve 1, complete reaction mixture (Assay I in Table 1): Curve 2, complete minus triose-P isomerase.

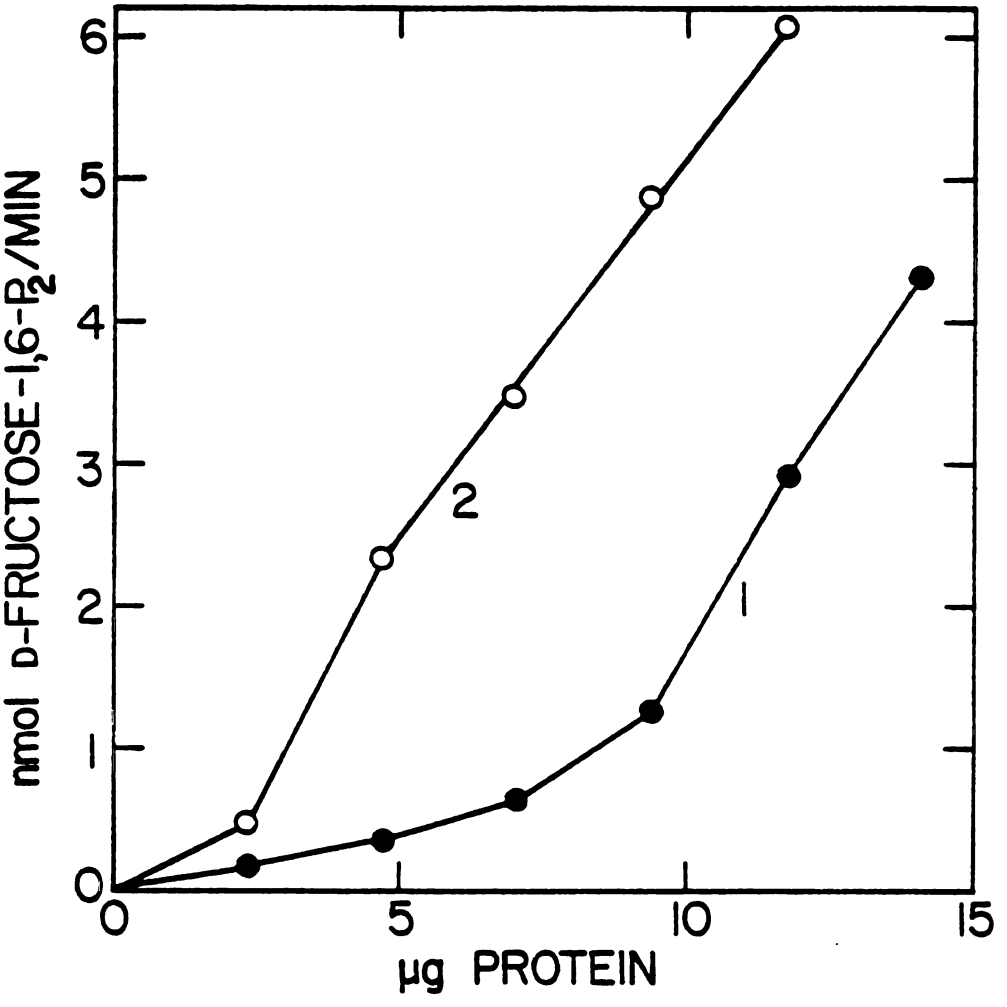

Figure 5

Direct demonstration of activation by fructose 1,6-bisphosphate. A direct demonstration of fructose-1,6-P_2-dependent activation of PP_i:F6P 1-phosphotransferase was achieved by following the rate of P_i formation using a glyceraldehyde-3-P dehydrogenase-coupled assay (Table 1, Assay II). This assay necessitates that the reagents have negligible amounts of P_i so that the initial absorbance of the reaction mixture could be kept low. Of the reagents in the assay mixture, commercially available fructose-6-P was found to contribute a significantly high amount of P_i. The commercially available biochemical reagent grade fructose-6-P can have from 0.5 to over 1.0 mole % P_i on prolonged storage. A 0.5 mole % P_i will contribute an absorbance of 0.6 to the initial value, thereby reducing the range of absorbance that is available to follow the progress of the reaction. Therefore, fructose-6-P was freed of its contaminant P_i by column chromatography through Sephadex G-10 (Figure 6).

Another precaution to be observed with the assay concerns the use of a suitable ADP reagent. The assay was inoperative unless ADP that was designated "vanadium-free" was used. The mechanism of inhibition of the assay by vanadium was not studied thoroughly. However, when vanadium-containing ADP was added to the assay mixture for PP_i:F6P 1-phosphotransferase by fructose-1,6-P_2 aldolase-triose-P isomerase-α-glycerol-P-dehydrogenase, no such inhibition was observed, suggesting that the vanadium inhibits either glyceraldehyde-3-P dehydrogenase, 3-phosphoglycerate kinase, or both. The inhibition by vanadium was not studied further, although it was known (142-145) that vanadium inhibits Na/K ATPase, and therefore the site of inhibition by

Figure 6. Elution profile of fructose-6-P and P_i by chromatography through Sephadex G-10. One-half mg of fructose-6-P was dissolved in H_2O to 1.7 ml and introduced to a Sephadex G-10 column (2.6 x 100 cm). The sugar phosphate was eluted with H_2O at room temperature at a linear flow rate of 2.5 cm/hr. The fructose-6-P was determined by enzymatic end-point assay using phosphoglucose isomerase and glucose-6-P dehydrogenase, whereas the P_i concentration was determined by enzymatic end-point assay using glyceraldehyde-3-P dehydrogenase and 3-phosphoglycerate kinase. Other details of the assay are in the text.

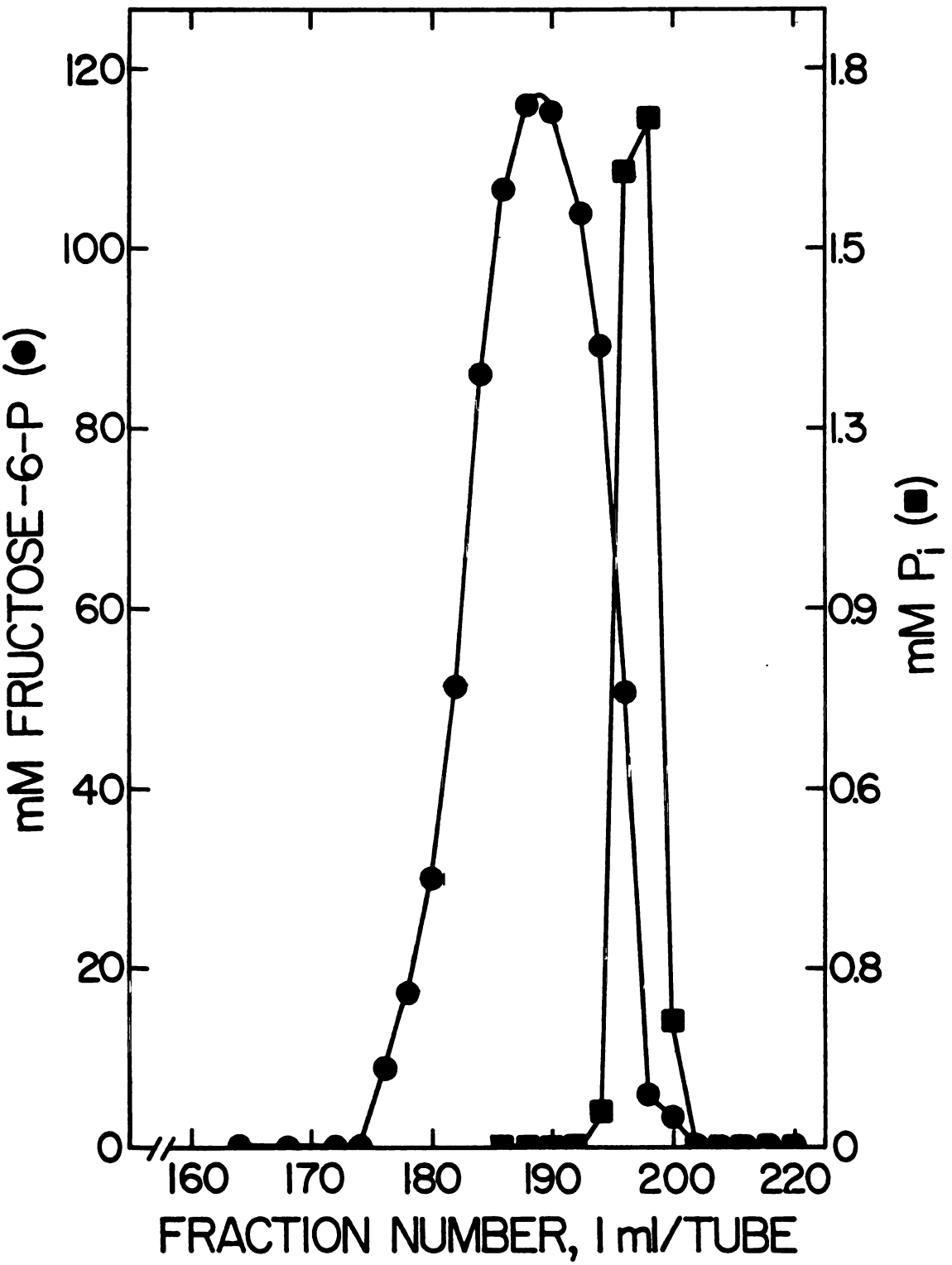
120
100
80
60
40
20
0
mM FRUCTOSE-6-P (●)
1.8
1.5
1.3
0.9
0.6
0.8
0
mM Pi (■)
160
170
180
190
200
220
FRACTION NUMBER, 1 ml/TUBE

Figure 6

vanadium may be the 3-phosphoglycerate kinase step of the enzymatic assay.

Having worked out the procedure for the assay that could monitor P_i formation, the experiment to demonstrate the effect of fructose-1,6-P_2 was conducted. In the presence of added 100 μM fructose-1,6-P_2, the velocity was maximal and was constant with time, whereas in its absence the velocity was initially slow and increased with time (as the fructose-1,6-P_2 product accumulated) to the same rate as that achieved with initially added fructose-1,6-P_2 (Figure 7). ATP, 1,3-diphosphoglycerate, or 3-phosphoglycerate, did not activate the PP_i:F6P 1-phosphotransferase when added to the reaction mixture during the fructose-1,6-P_2 aldolase-coupled assay. The conclusion was that micromolar concentrations of fructose-1,6-P_2 activated PP_i:F6P 1-phosphotransferase, causing a many-fold increase in the reaction velocity even at saturating levels of the substrates, fructose-6-P and PP_i.

Activation by glucose 1,6-bisphosphate. The glyceraldehyde-3-P dehydrogenase-coupled assay was not convenient for routine measurements of PP_i:F6P 1-phosphotransferase because of the need for P_i-free reagents and the inapplicability of the assay to crude extracts, which contain phosphatases. So the search for other possible activators was continued and the logical approach was to try other hexose bisphosphates. Glucose-1,6-P_2 was found able to replace fructose-1,6-P_2 as an activator in the aldolase-coupled assay (Table 1, Assay I). Glucose-1,6-P_2 (1.5 mM) activated about 20 fold, and the reaction velocity was proportional to PP_i:F6P 1-phosphotransferase concentration. Glucose-1,6-P_2 also activated PP_i:F6P 1-phosphotransferase in the

Figure 7. Activation of PP_i:F6P 1-phosphotransferase by fructose-1,6-P_2. Curve 1, complete reaction mixture (Assay II of Table 1) plus 0.10 mM fructose-1,6-P_2; Curve 2, complete reaction mixture (no fructose-1,6-P_2 added); Curve 3, blank (complete minus fructose-6-P and PP_i).

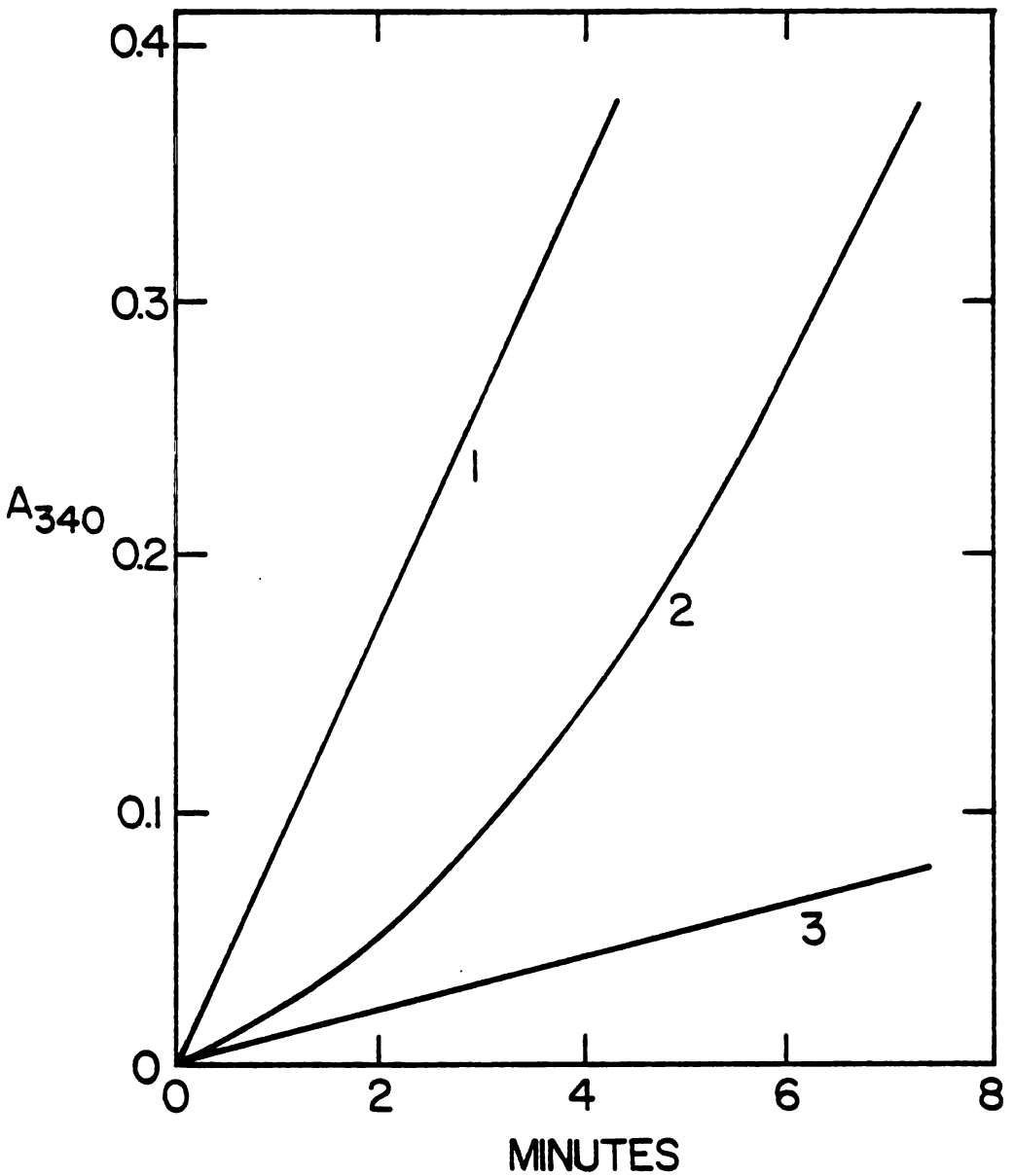

Figure 7

glyceraldehyde-3-P dehydrogenase-coupled assay (Table I, Assay II), thus abolishing the lag. Glucose-1,6-P_2 did not further activate the enzyme when the reaction was run in the reverse direction (Table 1, Assay III) because in this case fructose-1,6-P_2 served both as the substrate and the activator. Glucose-1,6-P_2 could not replace fructose-1,6-P_2 as the substrate.

Optimization of the Assays for PP_i:F6P 1-Phosphotransferase.

Forward reaction (standard assay). A preliminary determination of the K_A for glucose-1,6-P_2 indicated it to be about 0.5 mM. The magnesium ion required for half-maximal activity was 0.3 mM (Figure 8), and 6 mM Mg^{++} gave the highest V_{max} of the metal ions tested. The activity as a function of pH was maximal at about pH 7.8 in HEPES-NaOH buffer. One millimolar PP_i was optimal and the activity at 20 mM fructose-6-P approached the plateau for the saturation curve, which started to decrease beyond 30 mM. The addition of 0.4 mM EDTA (Figure 9) completely abolished the background activity, whereas the activity was constant in the presence of 6 mM Mg^{++} even when EDTA was present at 2 mM. In addition, it was determined that $(NH_4)_2SO_4$ from the coupling enzymes contributed an inhibitory effect. From the above data, the conditions for the tentative standard assay using the aldolase-coupled assay that was adopted was as follows: an assay mixture (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 1 mM Na EDTA, 20 mM Na fructose-6-P, 1.0 mM Na PP_i, 6.0 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 1.5 mM glucose-1,6-P_2, and rate-limiting amounts of PP_i:F6P

Figure 8. Mg^{++} concentration-velocity curve for PP_i:F6P 1-phosphotransferase. The assay mixture (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 20 mM Na fructose-6-P, 1.0 mM Na PP_i, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 1.5 mM glucose-1, 6-P_2 and 5 µl of the 100-fold purified enzyme. The velocity is expressed as nmol of fructose-1,6-P_2 formed per min.

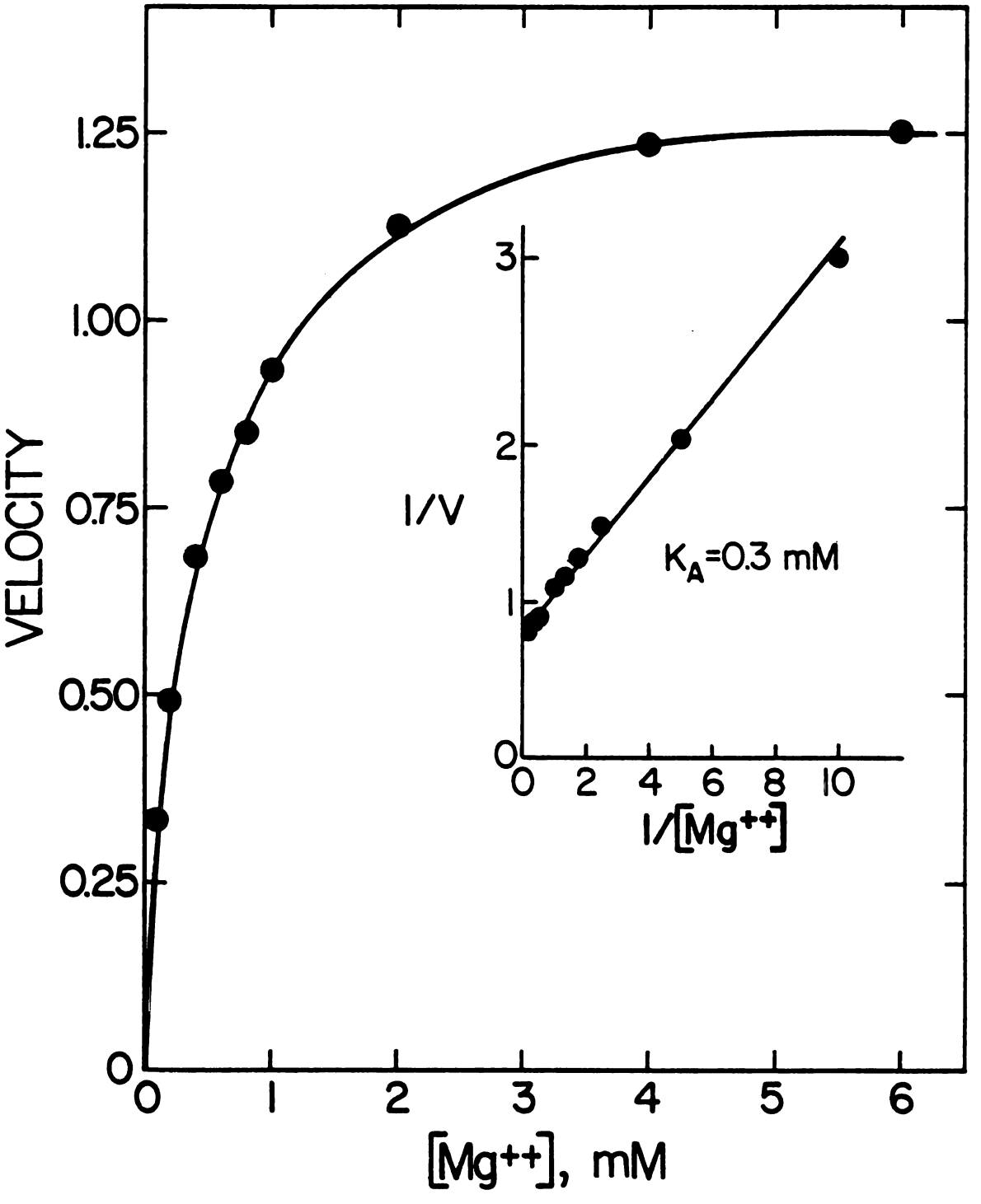
VELOCITY
1.25
1.00
0.75
0.50
0.25
0
0
1
2
3
4
5
6
[Mg++], mM
1/V
3
2
1
0
0
2
4
6
8
10
1/[Mg++]
K_A=0.3 mM

Figure 8

Figure 9. Absolute requirement of PP_i:F6P 1-phosphotransferase for divalent metal, Mg^{++}. The assay mixture (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 20 mM Na fructose-6-P, 1.0 mM Na PP_i, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 1.5 mM glucose-1,6-P_2 and 5 µl of the 100-fold purified enzyme. The activities were determined at no added Mg^{++}, and at 6 mM Mg^{++} at indicated Na EDTA concentrations.

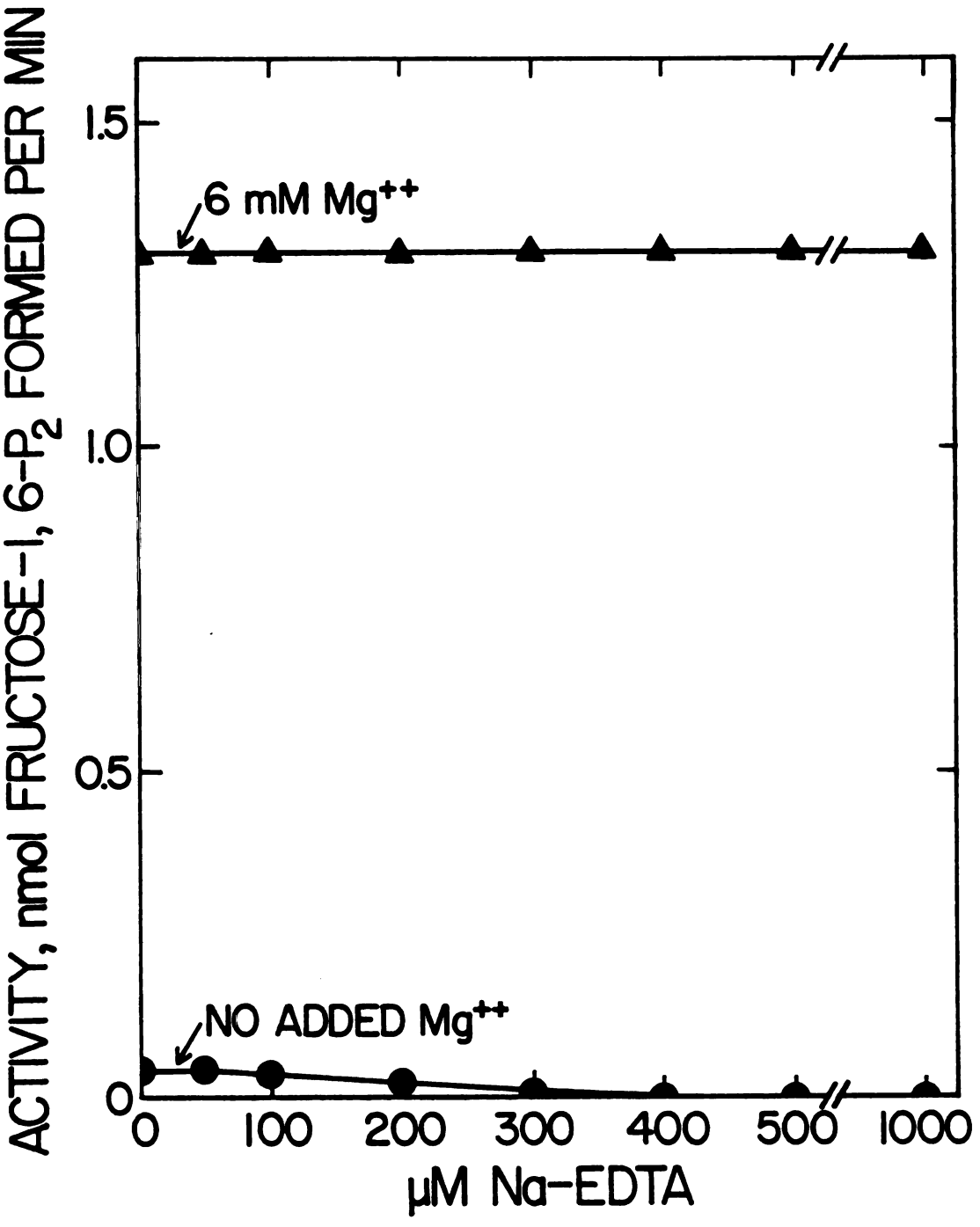
ACTIVITY, nmol FRUCTOSE-1, 6-P2 FORMED PER MIN
1.5
1.0
0.5
0
6 mM Mg++
NO ADDED Mg++
0
100
200
300
400
500
1000
μM Na-EDTA

Figure 9

1-phosphotransferase. The coupling enzymes were dialyzed against 1 mM EDTA at pH 7.8 to remove $(NH_4)_2SO_4$ or citrate which may be present.

Using the tentative standard assay described above, a specific activity of 0.018 unit of PP_i:F6P 1-phosphotransferase per mg protein was detected in the crude extract. This observed specific activity was an increase of about 40 times over that found when it was first detected, making the level of the enzyme about equal to that of ATP-dependent phosphofructokinase.

The final standard assay (Method A as described in Materials and Methods) was essentially the same as the above tentative standard assay except for halving the concentration of Na fructose-6-P to 10 mM and the use of 1 μM fructose-2,6-P_2 instead of 1.5 mM glucose-1,6-P_2, which were adopted after the discovery of fructose-2,6-P_2 activation (see below). Using fructose-2,6-P_2 as the activator, the specific activity of the enzyme in mung bean sprouts was about twice the apparent specific activity of the ATP-dependent phosphofructokinase.

Other assays for PP_i:F6P 1-phosphotransferase. Assay B (forward reaction alternative assay) and assay C (reverse reaction), as described in Materials and Methods, were also optimized by the approach described in the above section.

Determination of Inorganic Pyrophosphatase.

Determination of inorganic pyrophosphatase activity in the crude extract and in the partially purified PP_i:F6P 1-phosphotransferase was conducted employing Josse's modification (135) of Fiske and SubbaRow method for P_i determination. In the crude extract,

inorganic pyrophosphatase was apparently absent, with specific activity of less than 0.002 unit/mg protein (Table 4). Simmons and Butler (146) reported that mature seeds of maize have no inorganic pyrophosphatase, also. The subcellular localization of the inorganic pyrophosphatase in higher plants is in the chloroplast (146, 147).

Table 4 also shows that partially purified PP_i:F6P 1-phosphotransferase was devoid of inorganic pyrophosphatase activity.

Some Properties of the ATP-dependent Phosphofructokinase in Mung Beans.

The enzyme that is usually responsible for the formation of fructose-1,6-P_2 during glycolysis is the ATP-dependent phosphofructokinase, normally a regulatory enzyme. Since PP_i:F6P 1-phosphotransferase may also be involved in glycolysis, it was decided to determine some kinetic properties of the mung bean ATP-dependent phosphofructokinase for comparison with those of the pyrophosphate-dependent enzyme.

ATP-dependent phosphofructokinase was partially purified (20-fold) from mung beans by ammonium sulfate precipitation, chromatography on a Sephadex G-100 column, and two successive steps of column chromatography on DEAE-cellulose.

The pH dependence of the activity of the enzyme is shown in Figure 10. It is characterized by a nearly dome-shaped curve with an optimal pH range of 7.2 to 7.8. The saturation curve for fructose-6-P gave hyperbolic kinetics (the data will be presented in Figure 19). In contrast, ATP-dependent phosphofructokinase from other sources (muscle, brain, heart, liver, kidney cortex, sperm, carrots, Brussels sprouts, N. crassa, C. pasteurianum, E. coli and yeast) gave sigmoidal kinetics in the absence of an added allosteric effector (148). The effect of

Table 4. Assay for inorganic pyrophosphatase. The crude extract and partially purified PP_i:F6P 1-phosphotransferase (1.52 units/mg) were assayed for possible inorganic pyrophosphatase activity employing Josse's modification (135) of the Fiske and SubbaRow method. The assay mixtures were incubated for 20 min at 30°C. The sensitivity of the assay was 0.04 µmol of P_i. Therefore the specific activity of inorganic pyrophosphatase in the crude extract was less than 0.002 µmol of PP_i consumed per min per mg protein.

Sample	Volume Protein Preparation (µl)	Protein (mg)	P_i Liberated (µmol)	Specific Activity (U/mg protein)
Crude extract				
	10	0.15	0.00	<0.007
	20	0.30	0.000	<0.003
	30	0.45	0.00	<0.002
PP_i:F6P 1-Phosphotransferase				
	20	0.26	0.00	<0.004
	40	0.52	0.00	<0.002

Figure 10. Effect of pH on the activity of ATP-dependent phosphofructokinase. The reaction mixture (170 µl) contained 1.0 mM ATP, 10 mM fructose-6-P, 6 mM $MgCl_2$, 0.24 mM NADH, 0.2 unit of fructose-1, 6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 1.3 mU of the DEAE-cellulose II ATP-dependent phosphofructokinase preparation and 100 mM of the indicated buffer.

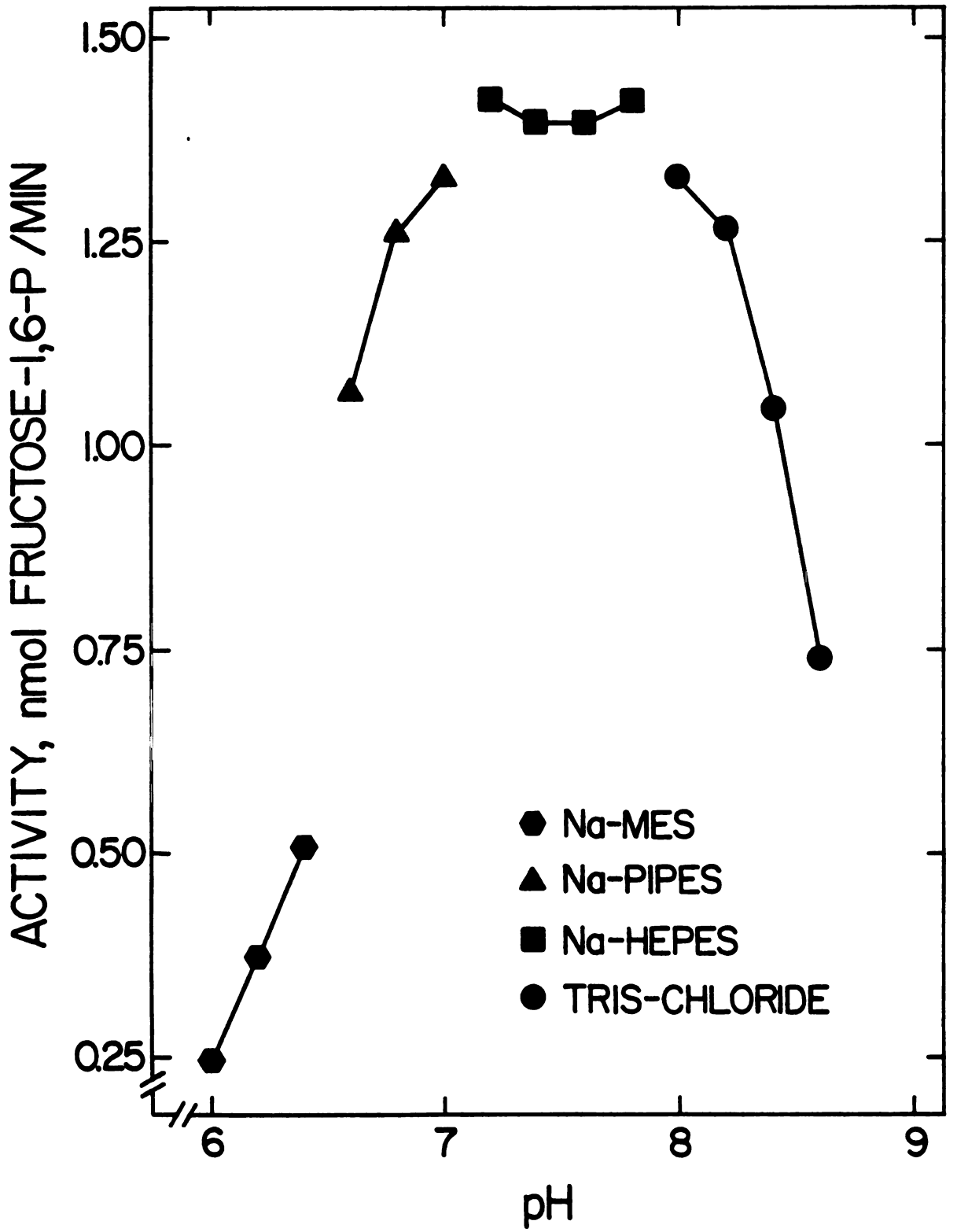
ACTIVITY, nmol FRUCTOSE-1,6-P /MIN
1.50
1.25
1.00
0.75
0.50
0.25
6
7
8
9
pH
Na-MES
Na-PIPES
Na-HEPES
TRIS-CHLORIDE

Figure 10

ATP concentration is shown in Figure 11. An increasing ATP concentration up to 2 mM stimulated the activity whereas a further increase was inhibitory. This behavior of inhibition by a high concentration of ATP was also observed for ATP-dependent phosphofructokinases from other sources (32, 149).

The effect of some metabolites on the activity of the ATP-dependent phosphofructokinase is shown in Table 5. Orthophosphate activated, whereas PP_i, AMP, ADP, citrate and phosphoenolpyruvate inhibited. The most pronounced inhibition was observed with phosphoenolpyruvate. In a separate experiment, it was observed that 10 μM phosphoenolpyruvate inhibited >99% (Figure 12). The phosphoenolpyruvate concentration that gave 50% inhibition was 2 μM, making phosphoenolpyruvate a very potent inhibitor of ATP-dependent phosphofructokinase. The potent inhibition by phosphoenolpyruvate has been previously observed for the ATP-dependent phosphofructokinase in peas (150). It should be recalled that phosphoenolpyruvate does not inhibit mung bean PP_i:F6P 1-phosphotransferase even at 1 mM.

Stability of PP_i:F6P 1-Phosphotransferase at Various Conditions of Storage.

It was observed initially that the partially purified enzyme lost activity on storage both at 4°C and at -20°C. To find a storage condition that would preserve the activity, varying storage temperatures and the addition of possible protecting reagents (glycerol, DTT, fructose-6-P and PP_i) were tried. The enzyme (in 10 mM Tris-acetate buffer - pH 7.3, 0.1 mM EDTA, 1.0 mM DTT, and 17 mM KCl) was stable (100% in 4 months) on storage at -20°C with added 20% (v/v) glycerol,

Figure 11. ATP concentration-velocity curve for mung bean ATP-dependent phosphofructokinase. The assay mixture (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.4), 10 mM fructose-6-P, 6.0 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 0.43 mU of DEAE-cellulose II ATP-dependent phosphofructokinase, and the indicated amounts of ATP.

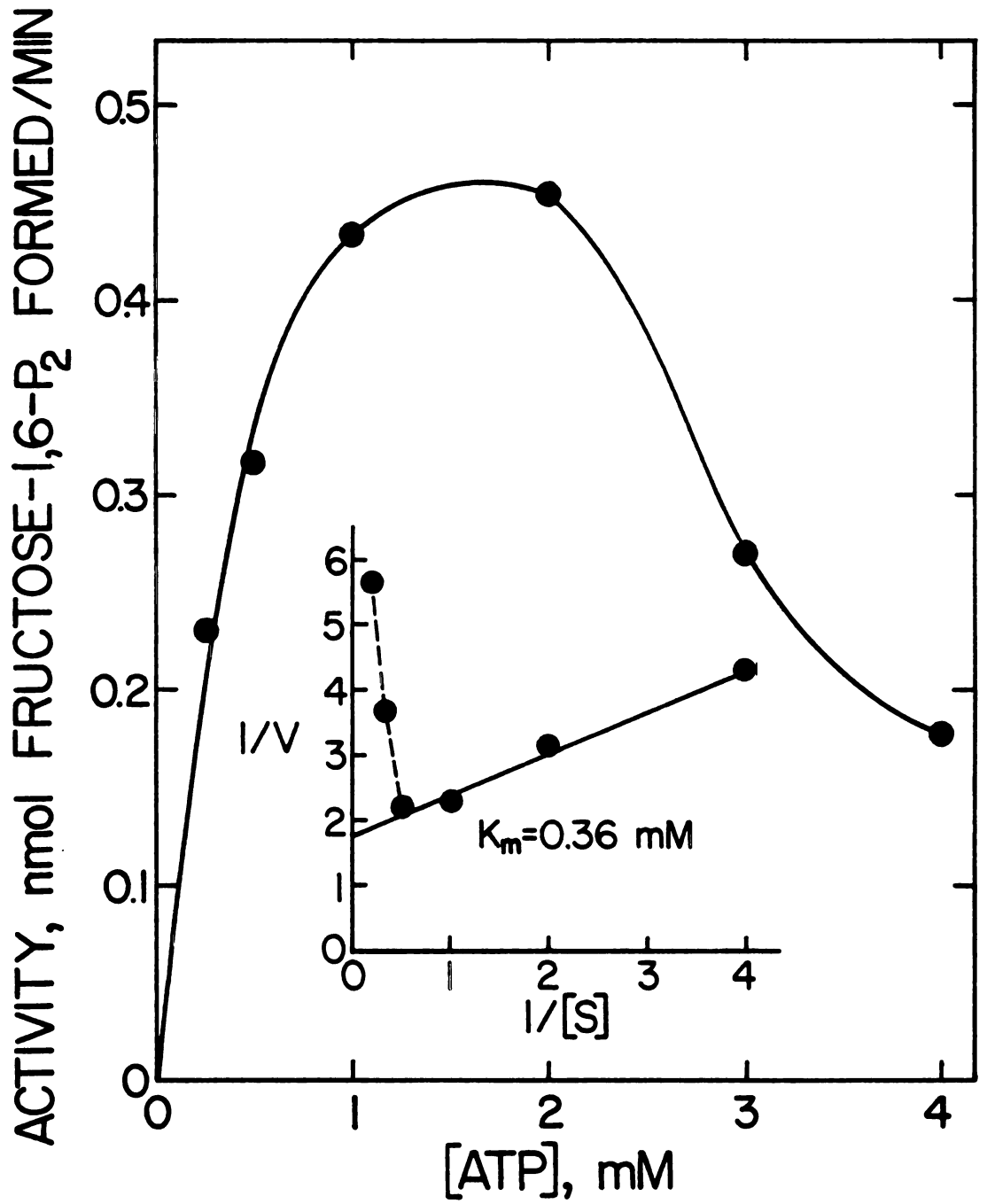
ACTIVITY, nmol FRUCTOSE-1,6-P2 FORMED/MIN
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
[ATP], mM
1/V
6
5
4
3
2
1
0
0
1
2
3
4
1/[S]
Km=0.36 mM

Figure 11

Table 5. Effect of some metabolites on ATP-dependent phosphofructokinase. The reaction mix (170 µl) contained 80 mM HEPES buffer (pH 7.4), 10 mM fructose-6-P, 1.0 mM ATP, 6 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase and 10 µl of the partially purified DEAE-cellulose II ATP-dependent phosphofructokinase.

Concentration of effector in assay Mixture	Activity (nmol fructose-1,6-P_2 formed/min)		Effect
	No effector	With effector	
2 mM P_i	0.131	0.179	37% Activation
2 mM PP_i	"	0.093	29% Inhibition
2 mM AMP	"	0.083	37% "
2 mM ADP	"	0.107	18% "
2 mM Citrate	"	0.098	25% "
2 mM Phosphoenolpyruvate	"	0.000	100% "

Figure 12. Effect of micromolar concentrations of phosphenolpyruvate on the activity of ATP-dependent phosphofructokinase. The assay mixture (170 µl) contained 80 mM HEPES-NaOH buffer (pH 7.4), 1 mM Na fructose-6-P, 0.5 mM ATP, 6.0 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, and 0.43 mU of the DEAE-cellulose II enzyme preparation.

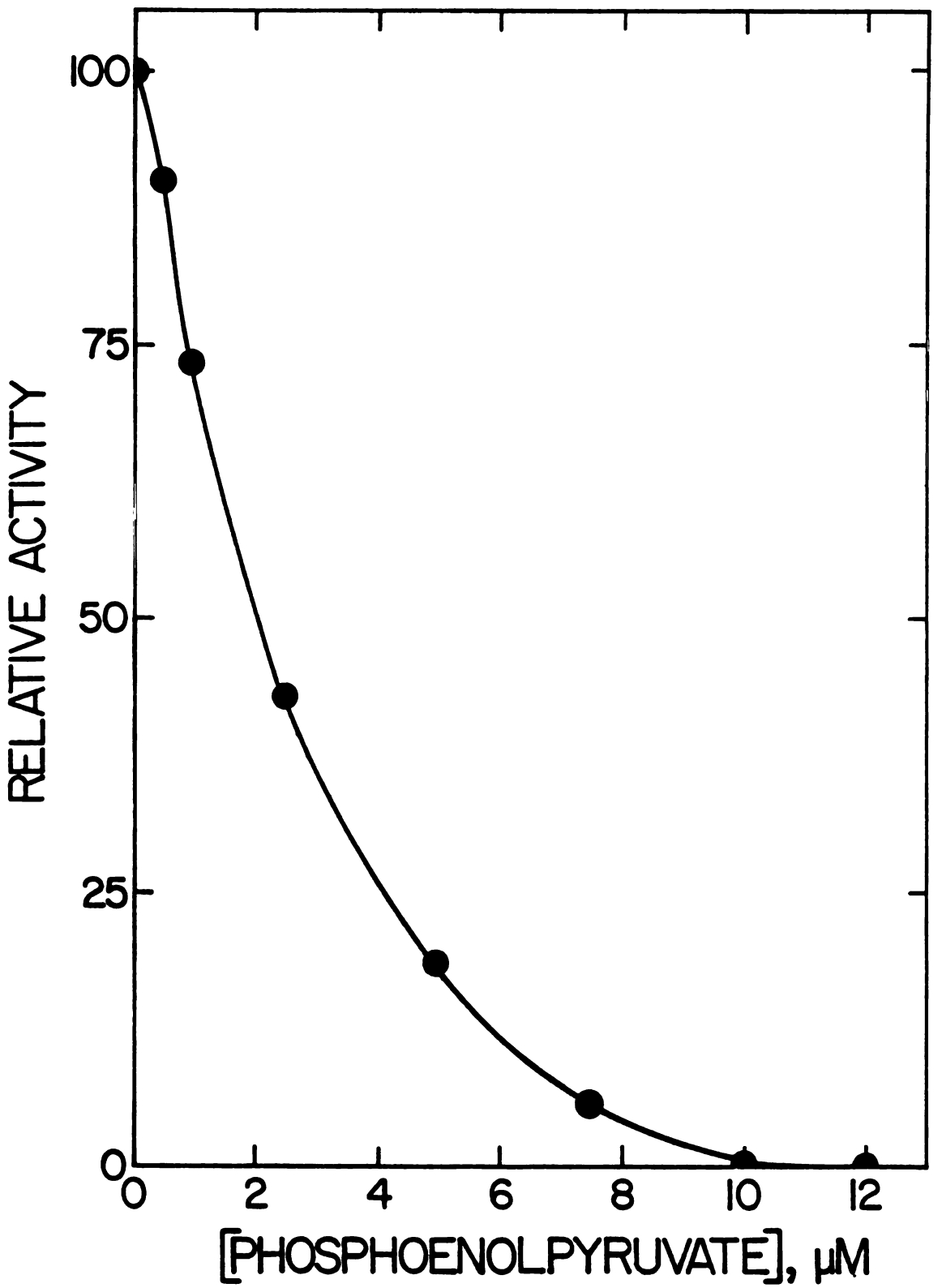
100
75
50
25
0
RELATIVE ACTIVITY
0
2
4
6
8
10
12
[PHOSPHOENOLPYRUVATE], μM

Figure 12

and at -80°C without added glycerol (Figure 13). It was unstable to storage at -20°C in the absence of glycerol (90% decrease in one week). At 4°C in the absence of glycerol, it retained its activity (100%) for about a week and then the activity decreased slowly (51% remaining after 13 weeks). At 4°C in the absence of glycerol, 17 mM PP_i did not provide any protection (44% remaining after 13 weeks). The addition of 170 mM fructose-6-P, at 4°C in the absence of glycerol, accelerated the decrease in activity (only 17% left compared to 70% for the control after 6 weeks). The addition of 16 mM DTT also accelerated the decrease in activity (only 14% left compared to 88% for the control after 3 weeks).

The storage-stability experiment established two storage conditions in which the enzyme retained its activity by 100% for at least 4 months (at -80°C, or at -20°C in the presence of 20% glycerol). In addition, the results suggested a possible stabilizing role of glycerol if it were added in the buffer during the purification of the enzyme, and indicated that DTT is deleterious to the stability of the enzyme.

Further Purification of the Enzyme and Elucidation of Additional Properties.

The enzyme was purified (670-fold) by a procedure described in the article by Sabularse and Anderson (139) and elaborated on in an article by Anderson and Sabularse (140). This purification was conducted before the effects of glycerol and DTT were fully appreciated. The purified enzyme was stored at -80°C. This enzyme preparation was used to determine the K_A for fructose-1,6-P_2; the activation effect of fructose-2,6-P_2 and its K_A; the effect on the K_m for fructose-6-P

Figure 13. Stability of PP_i:F6P 1-phosphotransferase at various conditions of storage. The enzyme preparation purified about 10-fold was from a pooled fraction after DEAE-cellulose chromatography. The enzyme was dialyzed against 10 mM Tris-acetate buffer (pH 7.3) containing 0.1 mM EDTA, 1.0 mM DTT and 17.0 mM KCL. Storage temperature and addition of glycerol, PP_i, fructose-6-P, and DTT were as indicated. For the frozen samples, separate vials were used for the determination of each data point. The assay mixture (170 µl) contained 20 mM fructose-6-P, 1.0 mM PP_i, 6 mM $MgCl_2$, 0.24 mM Na NADH, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase, 1.5 mM glucose-1,6-P_2, and 6.6 µg protein (5 µl, 1.0 mU of activity) of the enzyme preparation.

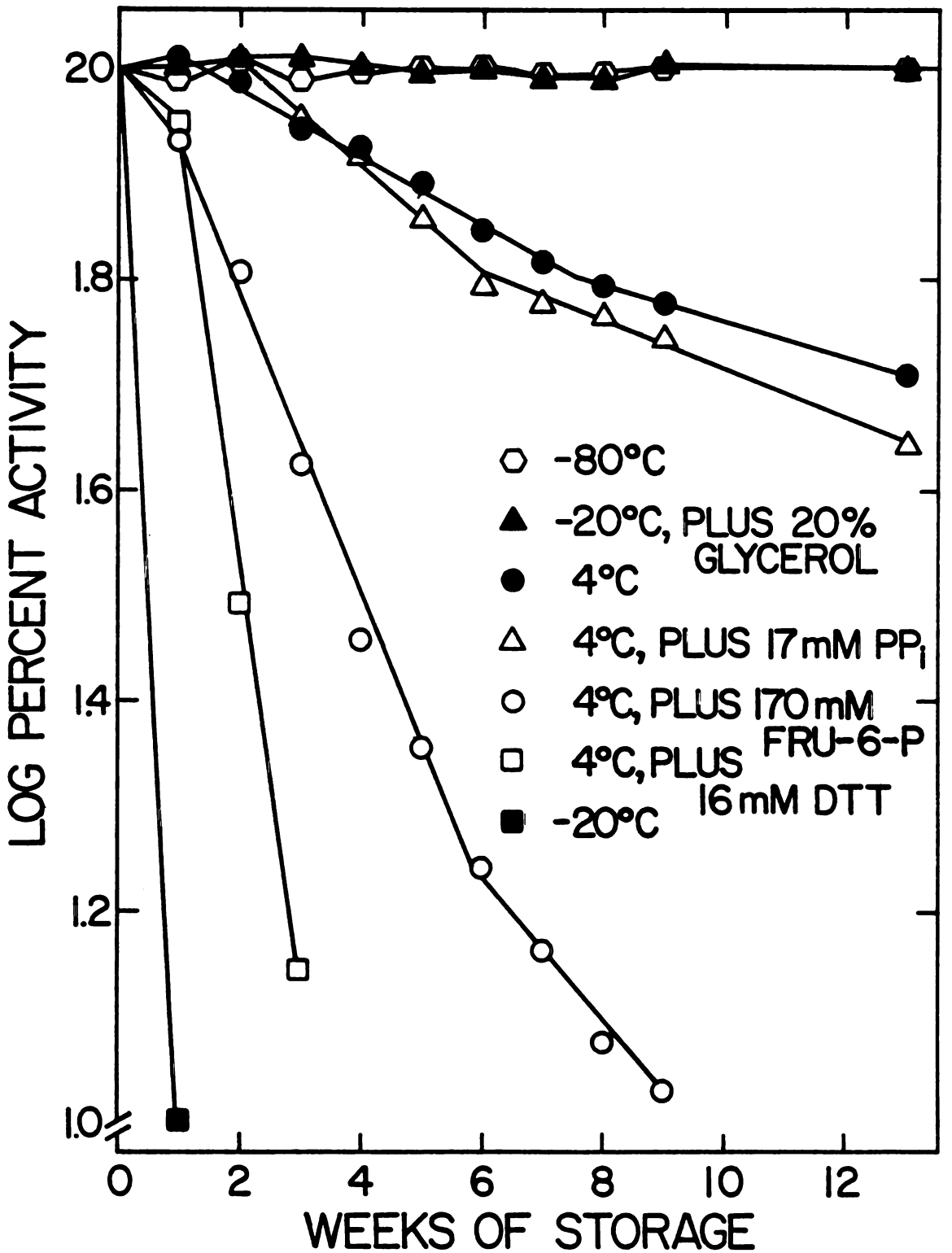
LOG PERCENT ACTIVITY
20
1.8
1.6
1.4
1.2
1.0
0
2
4
6
8
10
12
WEEKS OF STORAGE
-80°C
-20°C, PLUS 20% GLYCEROL
4°C
4°C, PLUS 17mM PP$_i$
4°C, PLUS 170mM FRU-6-P
4°C, PLUS 16mM DTT
-20°C

Figure 13

and the relative V_{max} by the activators; and as a reagent to determine the natural occurrence of fructose-2,6-P_2 in mung beans. A more extensive purification of the enzyme will be detailed in a later section of the thesis.

K_A for fructose 1,6-bisphosphate. The assay employed was a glyceraldehyde-3-P dehydrogenase-linked assay. Because fructose-1,6-P_2 is a product of the reaction, the accurate determination of its K_A required special instrumentation. The activity and the amount of the accumulated fructose-1,6-P_2 during the progress of the reaction were determined using a Gilford 2600 microprocessor-controlled spectrophotometer, programmed for the determination of enzyme kinetic parameters in a single assay mixture (151). The resulting graph (Figure 14) showed a hyperbolic response curve from which the K_A for fructose-1,6-P_2 was determined to be 17 μM.

K_A for glucose 1,6-bisphosphate. A fructose-1,6-P_2 aldolase-triose-P isomerase-α-glycerol-P dehydrogenase-linked assay was used, again employing the Gilford 2600 microprocessor-controlled spectrophotometer. The resulting graph (Figure 15) showed a hyperbolic response curve from which the K_A for glucose-1,6-P_2 was determined to be 0.4 mM.

Activation by fructose 2,6-bisphosphate and determination of its K_A. The discovery of fructose-2,6-P_2 as an activator of ATP-dependent phosphofructokinase in animal tissues (27, 31, 35) prompted us to test its effect on PP_i:F6P 1-phosphotransferase. Nanomolar amounts of fructose-2,6-P_2 were sufficient to activate PP_i:F6P 1-phosphotransferase to the same degree that was effected by μM amounts of fructose-1,6-P_2 or mM amounts of glucose-1,6-P_2. A

Figure 14. Determination of K_A of PP_i:F6P 1-phosphotransferase for fructose 1,6-bisphosphate. The assay employed was glyceraldehyde 3-P dehydrogenase-3-phosphoglyceric acid kinase-linked assay. The assay mixture (2000 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 1 mM EDTA, 10 mM fructose-6-P (P_i-free), 1 mM Na PP_i, 6.0 mM $MgCl_2$, 1.0 mM ADP (vanadium-free), 0.5 mM glyceraldehyde-3-P, 5 units of glyceraldehyde-3-P dehydrogenase, 0.5 mM NAD^+ and 3.5 µg protein of the enzyme preparation. The enzyme preparation was used to start the reaction.

The instrument used was a Gilford 2600 microprocessor-controlled spectrophotometer programmed for determination of kinetic parameters for enzymatic reaction in a single assay mixture (153). The instrument read and stored the data for instantaneous velocity and total fructose-1,6-P_2 formed every 2 sec for a 4-min program. Each data point was an integral of 11 readings resulting in 110 data points for 120 readings. A print out of the 110 data points of the recorded velocity (nmol fructose-1,6-P_2 formed per min) and the corresponding amounts of fructose-1,6-P_2 formed was obtained. A plot of every seventh data point of the set of velocity versus fructose-1,6-P_2 concentration was made along with the corresponding double reciprocal plot after subtracting the initial unactivated velocity from each of the velocity values to estimate the K_A. The inset is the corresponding double reciprocal plot of the fructose-1,6-P_2 concentration and the velocity.

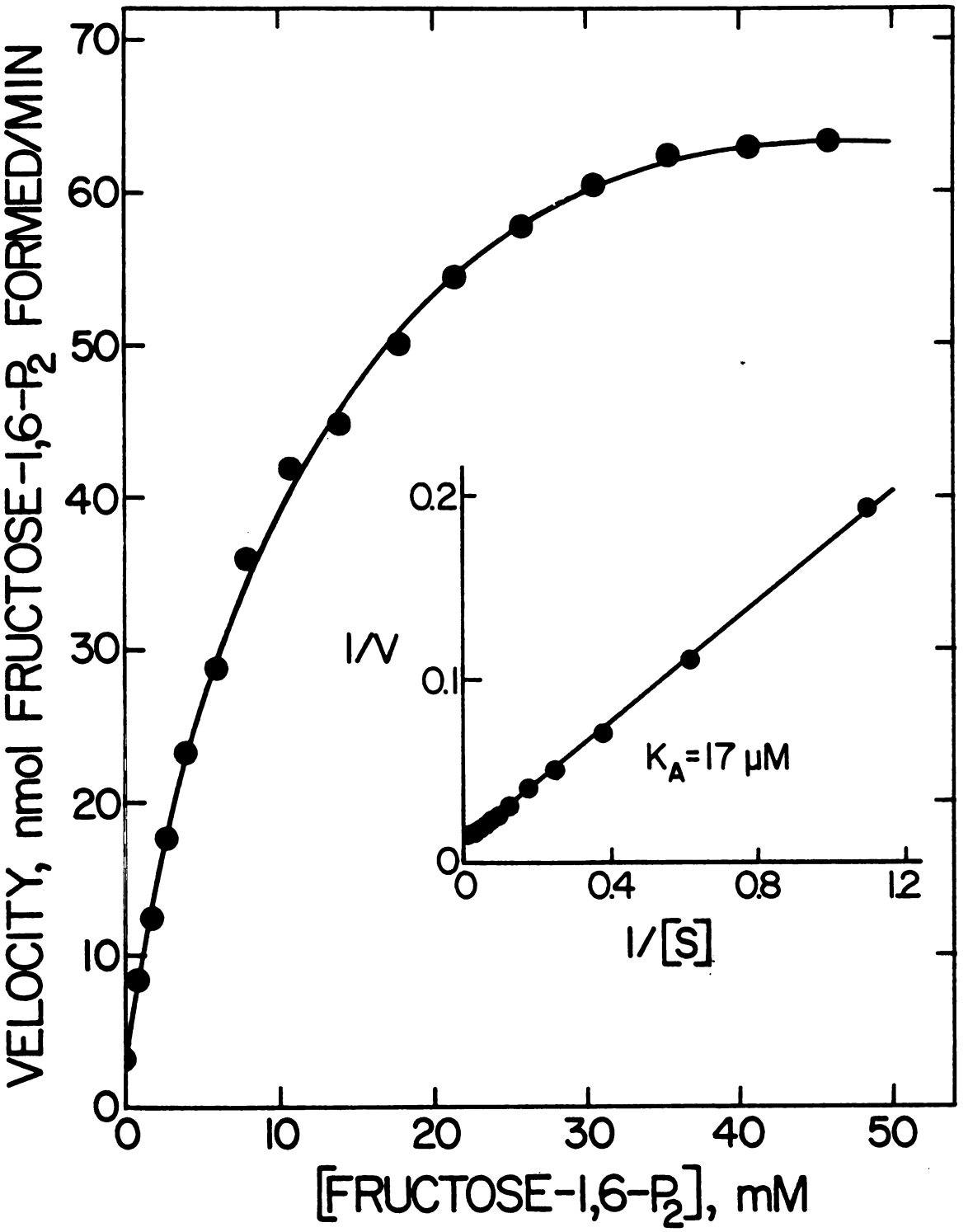
VELOCITY, nmol FRUCTOSE-1,6-P2 FORMED/MIN
[FRUCTOSE-1,6-P2], mM
0
10
20
30
40
50
60
70
1/V
1/[S]
0.2
0.1
0.4
0.8
1.2
KA=17 μM

Figure 14

Figure 15. Determination of K_A of PP_i:F6P 1-phosphotransferase for glucose-1,6-bisphosphate. The assay employ fructose-1,6-P_2 aldolase-triose-P isomerase-α-glycerol-P dehydrogenase-linked assay. The assay mixture (2000 µl) contained 80 mM HEPES-NaOH buffer (pH 7.8), 1 mM EDTA, 20 mM fructose-6-P, 1.0 mM Na PP_i, 6.0 mM $MgCl_2$, 0.3 mM NADH, 2.0 units of fructose-1,6-P_2 aldolase, 20 units of triose-P isomerase, 2.0 units of α-glycerol-P dehydrogenase, 2.1 µg protein of the enzyme preparation, and glucose-1,6-P_2 injected in increasing amount in the reaction mixture equipped with a stirrer.

The instrument used was a Gilford 2600 microprocessor-controlled spectrophotometer programmed for determination of kinetic parameters for enzymatic reaction in a single assay mixture (153). During the determination of the activity, the reaction mixture minus glucose-1,6-P_2 was read for the initial velocity (unactivated) collected as an average of 120 readings in 1 min. The glucose-1,6-P_2 (25.5 mM) was then injected into the reaction mixture at a flow rate predetermined to reach about 2.5 mM in 8 min. The instrument read and stored the data for the velocity and the corresponding concentration of the glucose-1,6-P_2 added, every 4 seconds for an 8-min program. Each data point was an integral of 11 readings resulting in 110 data points for 120 readings. A print out of the 110 data points of the recorded velocity (equivalent nmol of NADH oxidized per min) and the concentration of glucose-1,6-P_2 was collected. A plot of every 4th point of the set of velocity versus the glucose-1,6-P_2 concentration was made along with the double reciprocal plot after subtracting the initial unactivated velocity from each value, to estimate K_m. The inset is the corresponding double reciprocal plot of glucose-1,6-P_2 concentration and the velocity.

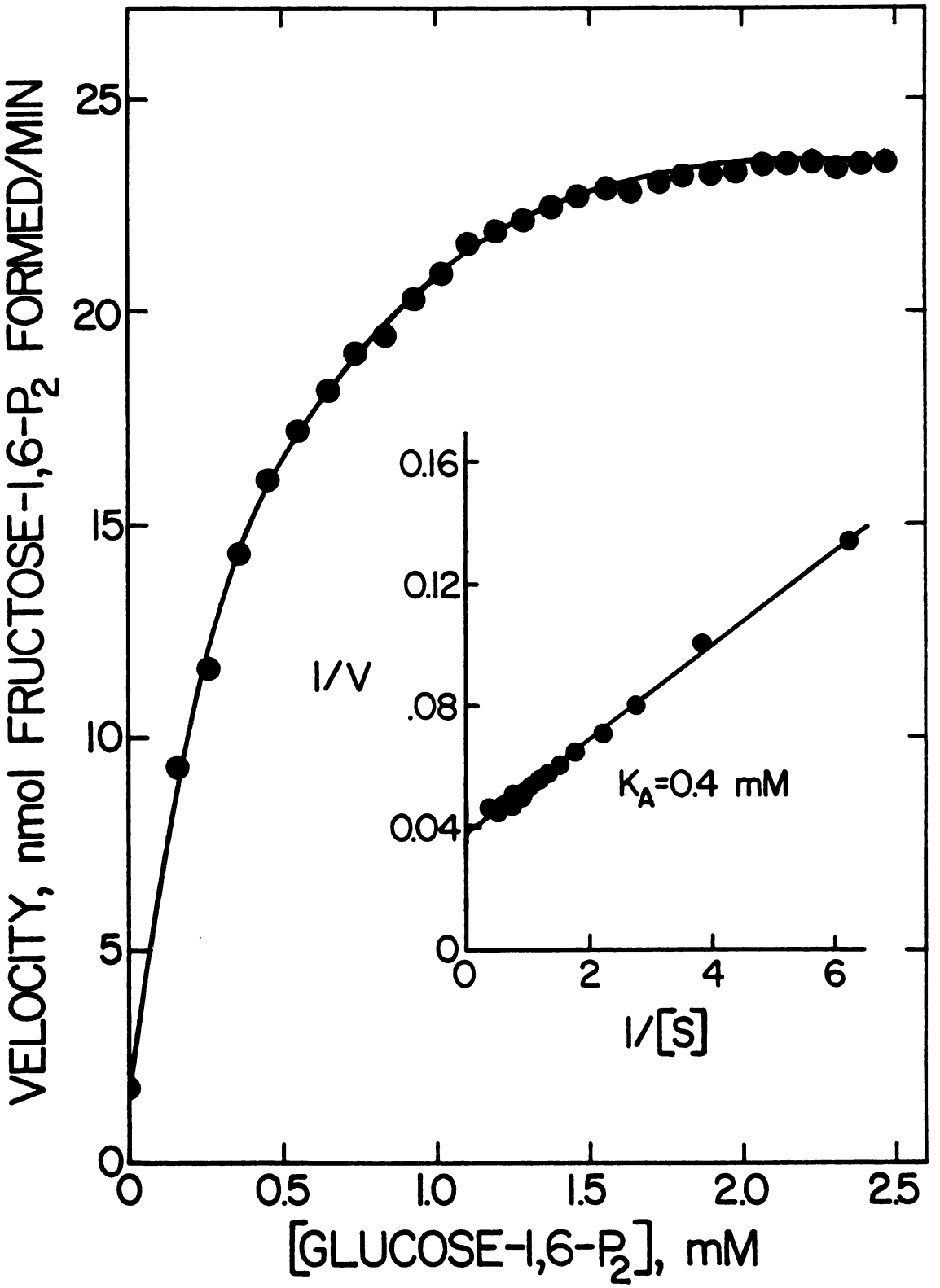
VELOCITY, nmol FRUCTOSE-1,6-P2 FORMED/MIN
25
20
15
10
5
0
0
0.5
1.0
1.5
2.0
2.5
[GLUCOSE-1,6-P2], mM
1/V
0.16
0.12
.08
0.04
0
0
2
4
6
1/[S]
KA=0.4 mM

Figure 15

plot of reaction velocity versus fructose-2,6-P_2 concentration (Figure 16) gave a hyperbolic curve from which the K_A for fructose-2,6-P_2 was determined to be 50 nM.

K_m for fructose-6-P and relative V_{max} of PP_i:F6P 1-phosphotransferase in the absence and in the presence of an activator. The hexose bisphosphates (glucose-1,6-P_2, fructose-1,6-P_2 and fructose-2,6-P_2) activate the enzyme both by decreasing the K_m for fructose-6-P and by increasing the V_{max}. Figure 17 shows a comparison of the saturation curve for fructose-6-P with no added activator and in the presence of a hexose bisphosphate activator. The corresponding double reciprocal plots (Figure 18) revealed that the enzyme follows hyperbolic kinetics both in the absence and in the presence of an activator. K_m values for fructose-6-P, and the relative V_{max} values, respectively, in the absence and presence of various activators, were as follows: no hexose bisphosphate, 20 mM, 1.0; glucose-1,6-P_2, 5.0 mM, 9.2; fructose-1,6-P_2, 0.56 mM, 9.2; and fructose-2,6-P_2, 0.12 mM, 12 mM, 15.2. The hexose bisphosphate concentration and the increase in the affinity (decrease in K_m) for fructose-6-P relative to that with no added activator, respectively, were as follows: 1.5 mM glucose-1,6-P_2, 4-fold; 100 μM fructose-1,6-P_2, 36-fold; and 1 μM fructose-2,6-P_2, 167-fold. It is significant to note that of the three hexose bisphosphates, fructose-2,6-P_2 is the most potent activator.

Figure 16. Determination of K_A of PP_i:F6P 1-phosphotransferase for fructose-2,6-P_2. The assay employed was as in the standard assay (Method A) except that varying concentrations of fructose-2, 6-P_2 were added. Enzyme protein amounting to 60 ng was used in each assay mixture. The inset is the corresponding double reciprocal plot of the fructose-2,6-P_2 concentration and the velocity after subtracting the initial unactivated velocity from each of the velocity values.

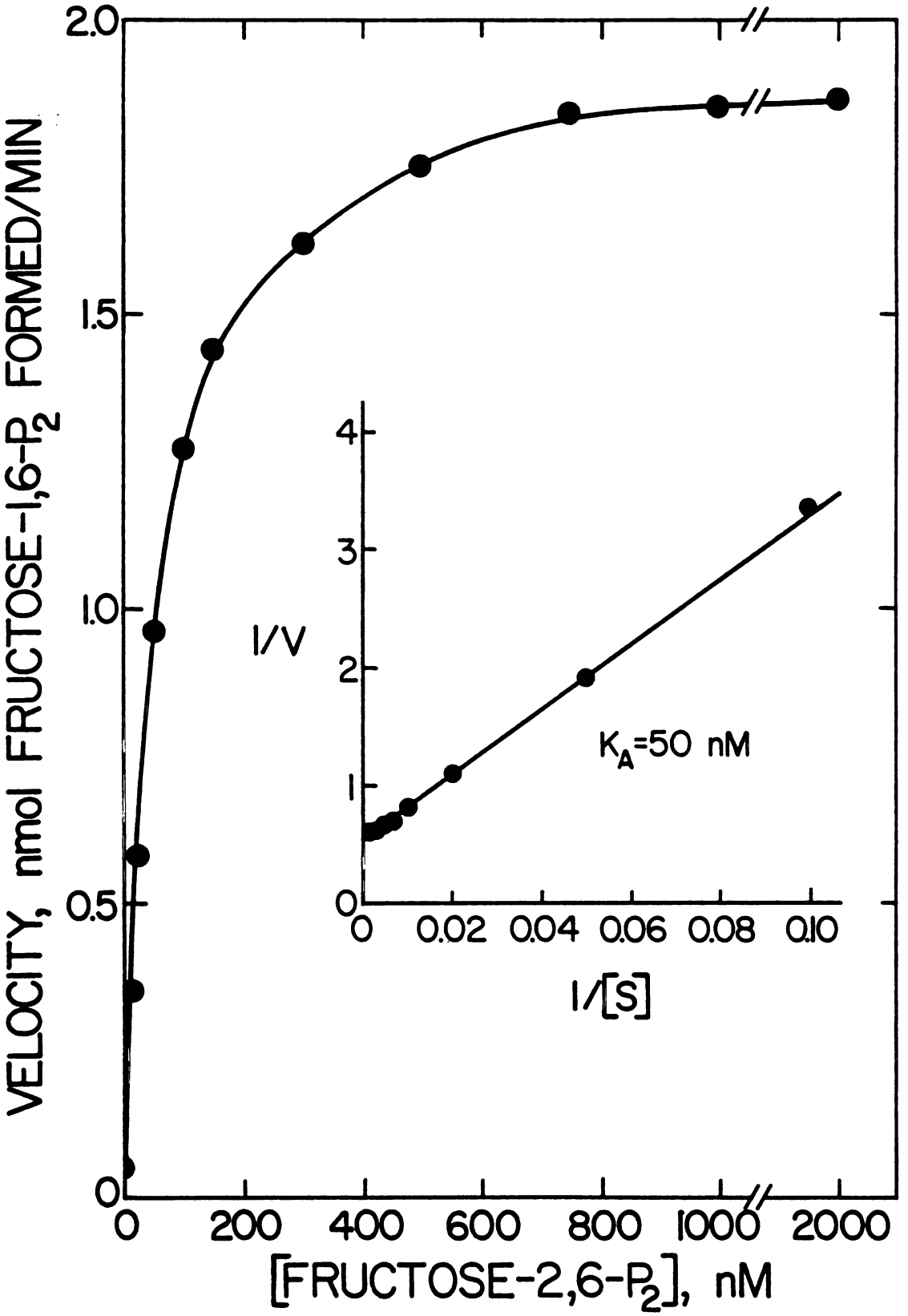
2.0
1.5
1.0
0.5
0
VELOCITY, nmol FRUCTOSE-1,6-P2 FORMED/MIN
0
200
400
600
800
1000
2000
[FRUCTOSE-2,6-P2], nM
1/V
4
3
2
1
0
0
0.02
0.04
0.06
0.08
0.10
1/[S]
KA=50 nM

Figure 16

Figure 17. Effect of fructose-2,6-P_2, fructose-1,6-P_2 and glucose-1,6-P_2 on the kinetic constants (K_m and V_{max}) of PP_i:F6P 1-phosphotransferase. Velocity was expressed as nmol of fructose-1,6-P_2 formed per min per 64 ng protein. Other details are given in the text. Double reciprocal plots are shown in Figure 18. For no activator, glucose-1,6-P_2 as the activator and fructose-2,6-P_2 as the activator, the activity was determined as in standard assay (Method A) with modifications indicated. For fructose-1,6-P as the activator, the activity was determined as in the alternative assay (Method B) with modifications indicated.

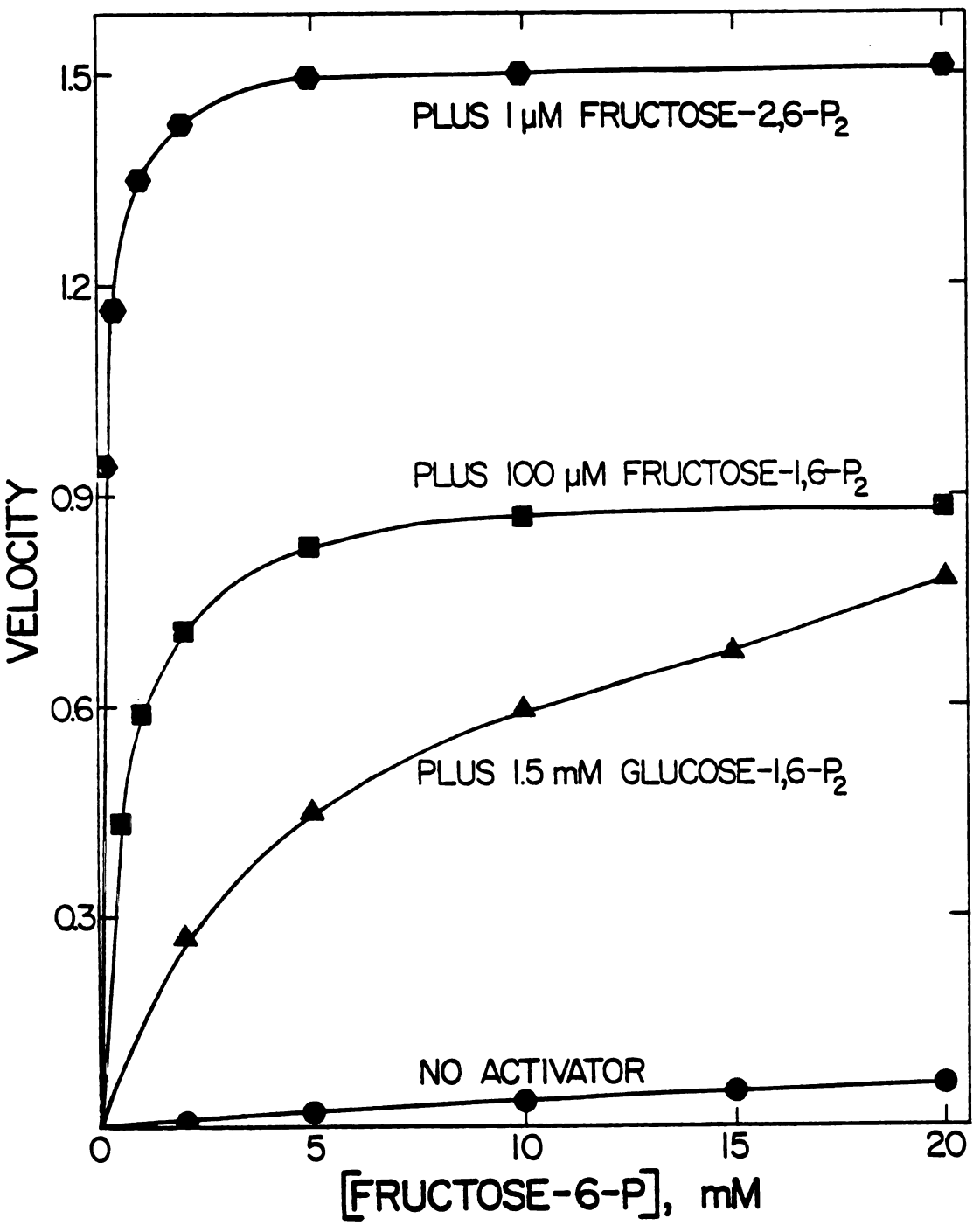
1.5
1.2
0.9
0.6
0.3
VELOCITY
PLUS 1 μM FRUCTOSE-2,6-P2
PLUS 100 μM FRUCTOSE-1,6-P2
PLUS 1.5 mM GLUCOSE-1,6-P2
NO ACTIVATOR
0
5
10
15
20
[FRUCTOSE-6-P], mM

Figure 17

Figure 18. Double reciprocal plots of the saturation curves for fructose-6-P in the absence and presence of various activators (Data from Figure 16).

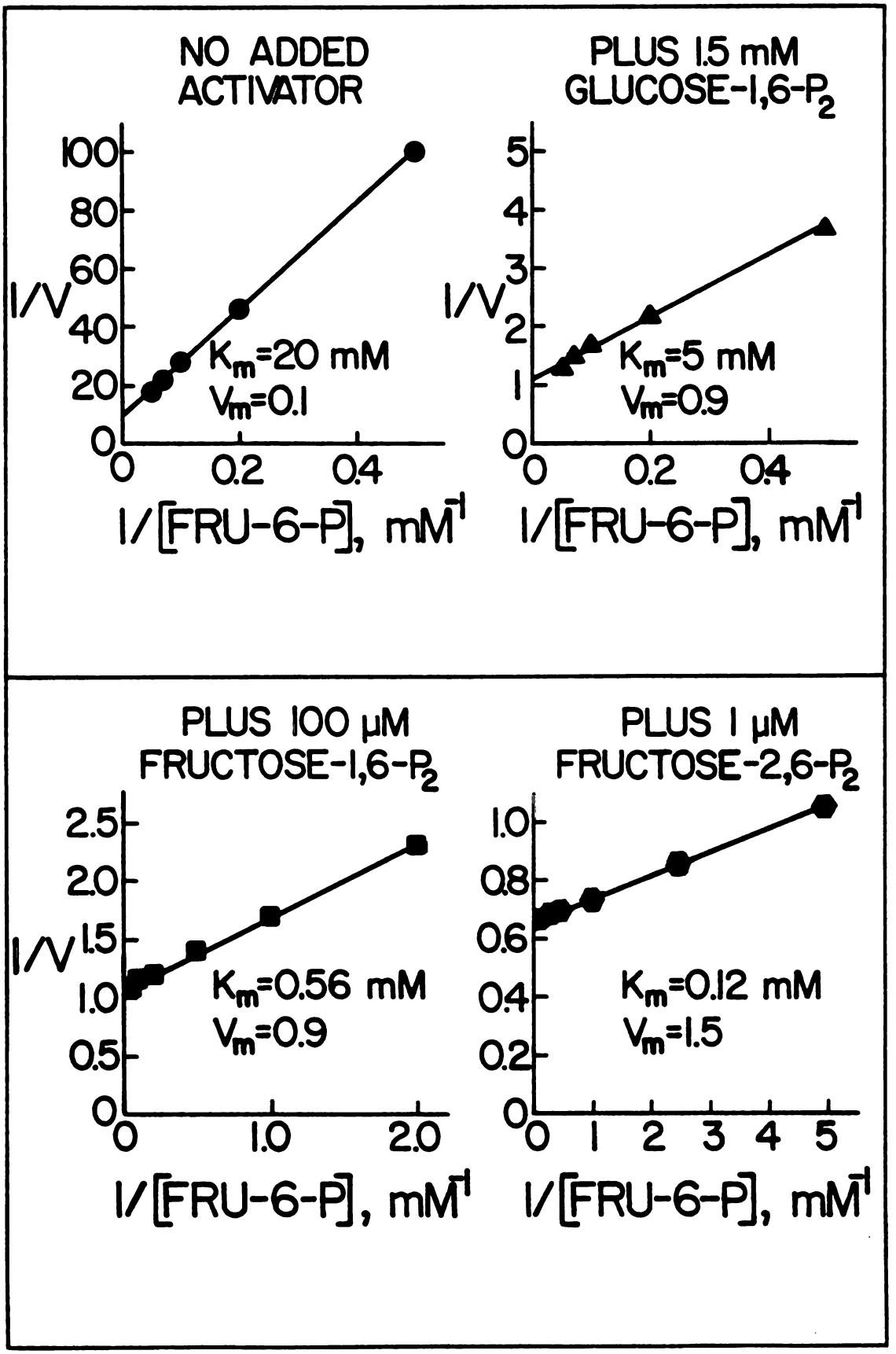
NO ADDED ACTIVATOR
PLUS 1.5 mM GLUCOSE-1,6-P2
1/V
Km=20 mM
Vm=0.1
Km=5 mM
Vm=0.9
1/[FRU-6-P], mM-1
PLUS 100 µM FRUCTOSE-1,6-P2
PLUS 1 µM FRUCTOSE-2,6-P2
Km=0.56 mM
Vm=0.9
Km=0.12 mM
Vm=1.5

Figure 18

Effect of Fructose 2,6-Bisphosphate on ATP-dependent Phosphofructokinase.

Fructose-2,6-P_2 had no apparent effect on the partially purified ATP-dependent phosphofructokinase from mung beans, which contrasts markedly with its recently reported potent activation of this enzyme in animal tisues (25, 30, 33). Plots of velocity versus fructose-6-P concentration yielded hyperbolic curves both in the absence and presence of 1.0 μM fructose-2,6-P_2 (superimposable curves; Figure 19). In both cases, the K_m for fructose-6-P was 0.9 mM and the V_{max} was 147 nmol of fructose-1,6-P_2 formed per min per milligram of protein. Subsequent to the report of Sabularse and Anderson (139), it was reported by Miernyk and Dennis (49) that in the case of the ATP-dependent phosphofructokinase from developing castor bean, the plastid ATP-dependent phosphofructokinase isozyme was activated by fructose-2,6-P_2 but only at pH 7.0. No activation by fructose-2,6-P_2 was observed at pH 8.0 and the cytoplasmic ATP-dependent phosphofructokinase isozyme was not activated either at pH 8.0 or 7.0. In the case of the mung bean ATP-dependent phosphofructokinase, no activation by fructose-2,6-P_2 was observed even at pH 7.0.

Isolation and Identification of Fructose 2,6-Bisphosphate from Mung Beans.

Chromatography of a mung bean extract on an anion-exchange column yielded a gradient-elution profile with two peaks in the hexose bisphosphate region (Figure 20A). The first peak was identified as

Figure 19. Substrate saturation curve for mung bean ATP-dependent phosphofructokinase. Two sets of data are shown; in the absence (●) and presence (□) of 1.0 μM fructose-2,6-P_2 (identical curves). The 8-fold purified enzyme (10.5 μg of protein) was used in each case. Velocity is expressed as nmol of fructose-1,6-P_2 formed per minute per 10.5 μg of protein.

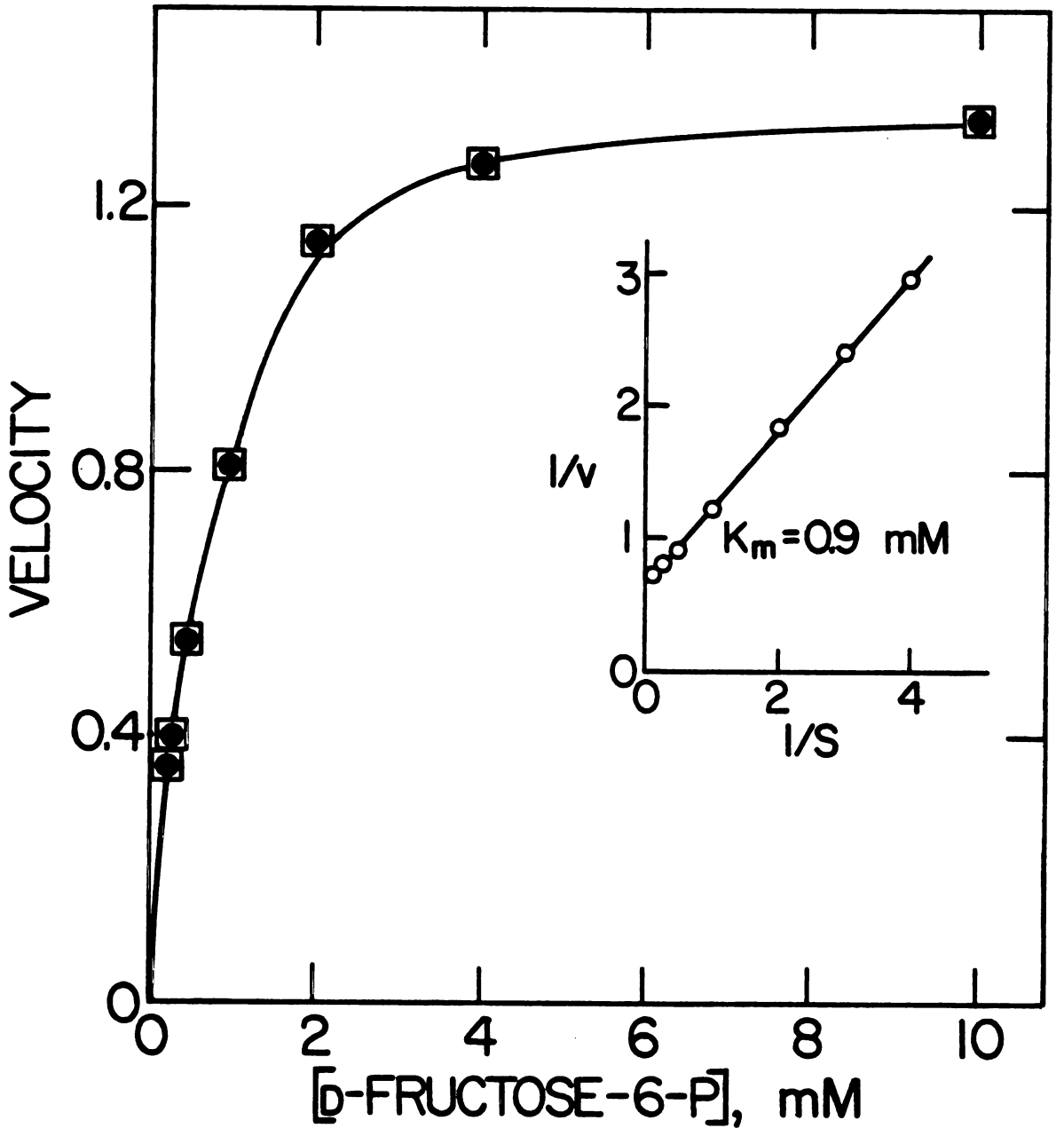
VELOCITY
1.2
0.8
0.4
0
0
2
4
6
8
10
[D-FRUCTOSE-6-P], mM
1/v
3
2
1
0
0
2
4
1/S
K_m=0.9 mM

Figure 19

Figure 20. Chromatographic evidence for the occurrence of fructose 2,6-bisphosphate in mung beans.

A, a mung bean extract was prepared and chromatographed on Bio-Rad AG 1-X8 as described in Materials and Methods.

B, a synthetic mixture of authentic fructose-1,6-P_2 and fructose-2,6-P_2 was chromatographed as in A.

Fructose-1,6-P_2 (○) was determined with an enzymatic end-point assay initiated by fructose-1,6-P_2 aldolase, whereas fructose-2,6-P_2 (●) was measured by its ability to activate PP_i:F6P 1-phosphotransferase (see Materials and Methods).

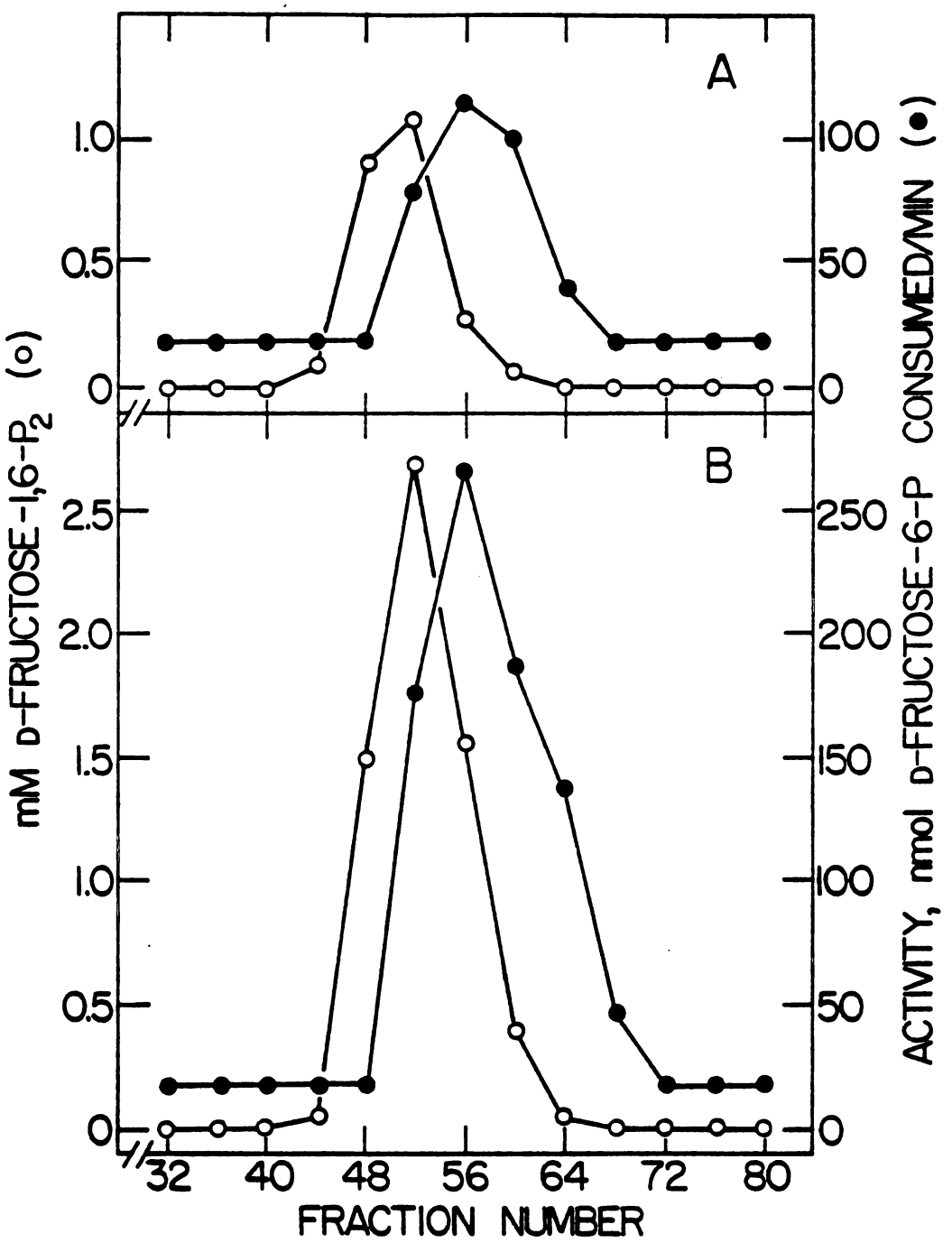
A
B
1.0
0.5
0
2.5
2.0
1.5
1.0
0.5
0
100
50
0
250
200
150
100
50
0
32
40
48
56
64
72
80
FRACTION NUMBER
mM D-FRUCTOSE-1,6-P2 (o)
ACTIVITY, nmol D-FRUCTOSE-6-P CONSUMED/MIN (•)

Figure 20

fructose-1,6-P_2 by its reaction in a coupled enzymatic assay initiated by fructose-1,6-P_2 aldolase. The second peak was located by its ability to activate PP_i:F6P 1-phosphotransferase and was identified as fructose-2,6-bisphosphate as described below.

Anion exchange chromatography of an authentic mixture of fructose 1,6-P_2 and fructose-2,6-P_2 (Figure 20B) yielded an elution profile similar to that observed with the mung bean extract (Figure 20A). The unknown activator in fraction 56 (Figure 20A) also behaved like authentic fructose-2,6-P_2 with respect to its stability in 0.25 N NaOH at 90°C and its room temperature lability at pH 2.5 (Table 6), thus locating one of the phosphate groups at carbon 2, assuming a ketohexose bisphosphate (25, 31).

The unknown activator was further identified as fructose-2,6-P_2 following a scaled-up procedure in which fructose-1,6-bisphosphatase treatment and a second anion-exchange chromatography step were used to separate the activator from fructose-1,6-P_2. The activator was further concentrated by lyophilization, subjected to mild acid hydrolysis (pH 2.5 at room temperature for 30 minutes), and the hydrolysis product was identified as fructose-6-P by specific coupled enzyme assays employing (i) D-fructose-6-phosphate isomerase (EC 5.3.1.9) and D-glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and (ii) phosphofructokinase (EC 2.7.1.11), D-fructose-1,6-bisphosphate aldolase, triose-P isomerase, and α-glycerol-P dehydrogenase.

Table 6. Comparison of the Mung Bean Natural Activator and Authentic Fructose 2,6-Bisphosphate with Respect to Acid- and Base-stability.

Treatment	Enzyme activity[a] in the presence of:	
	Fructose-2,6-P_2 (1 μM)	Natural activator
None	100	100
Acid hydrolysis[b]	0	0
Base hydrolysis[c]	100	100

[a]Activity is expressed as percent of the velocity observed in the standard assay using 3.1 milliunits of PP_i:F6P 1-phosphotransferase and the modifications indicated.

[b]pH 2.5 (adjusted with HCl) at 24°C for 30 min., then neutralized with NaOH.

[c]NaOH (0.25 N) at 90°C for 30 min., then neutralized with HCl.

Comparison of the Three Methods of Protein Determination.

The pooled fractions from various steps of purification of PP_i:F6P 1-phosphotransferase were assayed for protein using the method of Whitaker and Granum (134), Lowry et al. (136), and Bradford (137). The result showed a close agreement on the protein values (Table 7). The Whitaker and Granum method, a new procedure that was reported in 1980, has the advantage of being non-destructive, quick, and economical. However, the method is only 40% as sensitive as those of Lowry et al. and of Bradford. On the basis of these considerations the Whitaker and Granum method was adopted as the protein assay for subsequent protein determinations during the purification steps. Towards the later stages of purification, when the protein becomes very dilute, the microassay of Bradford may be used.

Final Purification and Partial Separation of Two Forms of PP_i:F6P 1-Phosphotransferase and Characterization of Their Properties.

Final purification. Because DTT accelerated the time-dependent decrease in the activity of the enzyme upon storage at 4°C and glycerol stabilized the activity of the enzyme on storage at -20°C, the following changes from earlier purifications were made: DDT was not added to the buffers starting with the grinding buffer, and 10% glycerol was added to the buffer after the ammonium sulfate precipitation step. The entire purification procedure is described in detail in the Materials and Methods section. The elution profiles of the enzyme and protein during the chromatography steps are shown as follows: Figure 21, DEAE-cellulose; Figure 22, phosphocellulose I; Figure 23, phosphocellulose II; and Figure 24, Bio-Gel A-1.5m. The

Table 7. Comparison of Three Methods of Protein Determination.

Purification Step	Activity[a]	Method								
		Whitaker and Granum			Lowry et al.			Bradford		
	(unit/ml)	Protein (mg/ml)	Sp. Act. (mU/mg)	Fold	Protein (mg/ml)	Sp. Act. (mU/mg)	Fold	Protein (mg/ml)	Sp. Act. (mU/mg)	Fold
Crude extract	0.150	8.30	18.1	1.0	9.41	15.9	1.0	10.5	14.3	1.0
30-45% $(NH_4)_2SO_4$ ppt.	2.81	66.1	42.5	2.4	87.1	32.3	2.0	65.1	43.2	3.0
DEAE-cellulose pH 7.3, 0-320 mM KCl gradient	6.31	35.1	180	10	43.3	146	9.2	42.6	148	10
Bio-Gel A-1.5m pH 7.3	4.61	11.0	419	23	14.5	318	20	12.7	363	25
Phosphocellulose I pH 6.5, 17 mM $Na_4P_2O_7$ elution	1.98	0.958	2070	114	1.14	1740	109	0.994	1990	139

[a]Activity of PP_i:F6P 1-Phosphotransferase was assayed by Method A (forward reaction, standard assay) except that the fructose-6-P was 20 mM and the activator was glucose-1,6-P_2.

Figure 21. DEAE-cellulose chromatography of PP_i:F6P 1-phosphotransferase. The protein was eluted by a linear gradient of KCl in the eluting buffer. All other details are given in the text.

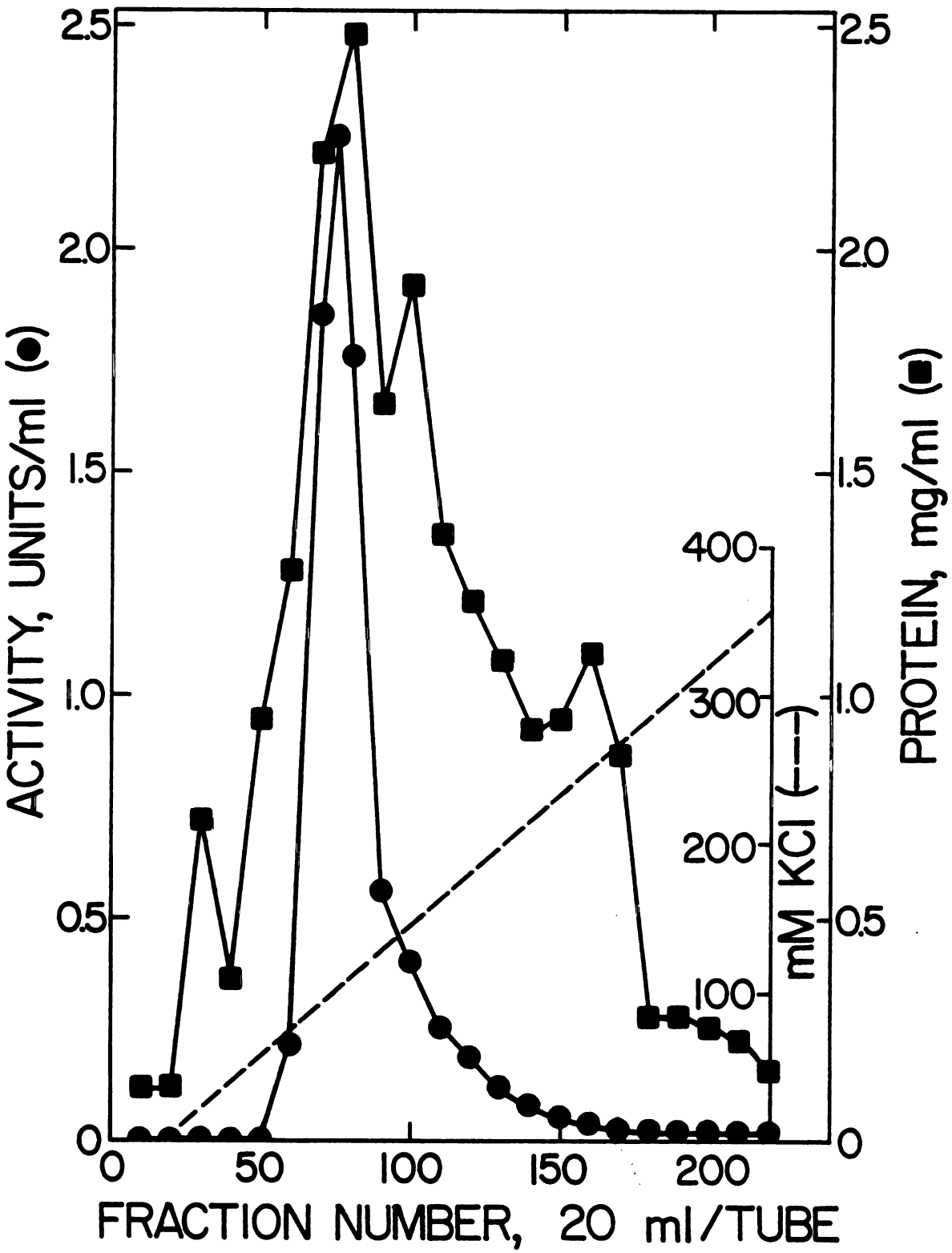
2.5
2.0
1.5
1.0
0.5
0
ACTIVITY, UNITS/ml (●)
PROTEIN, mg/ml (■)
400
300
200
100
mM KCl (---)
0
50
100
150
200
FRACTION NUMBER, 20 ml/TUBE

Figure 21

Figure 22. Phosphocellulose I Chromatography of PP_i:F6P 1-phosphotransferase. $Na_4P_2O_7$ (34 mM) was included in the eluting buffer. All other details are given in the text.

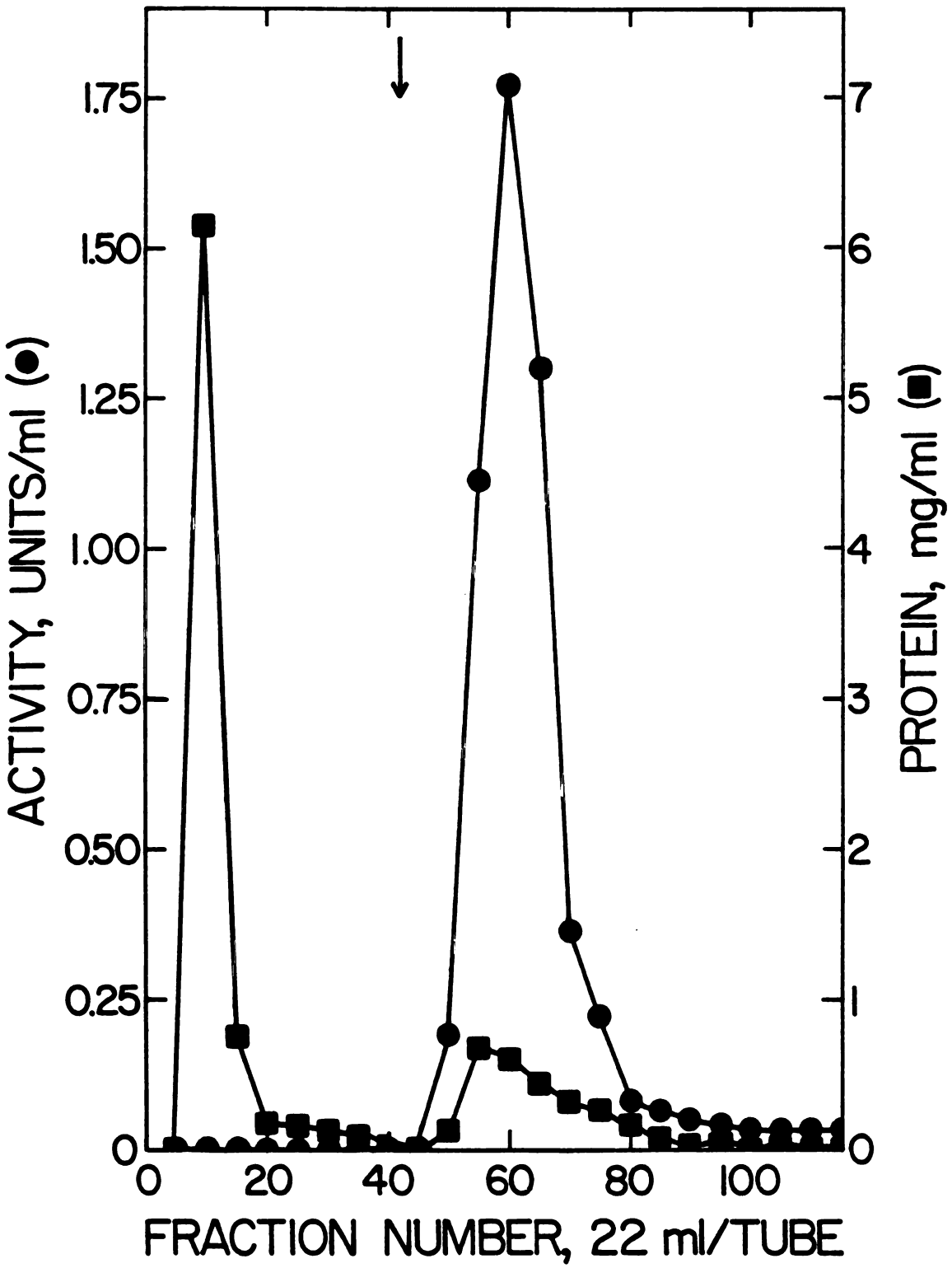
ACTIVITY, UNITS/ml (●)
PROTEIN, mg/ml (■)
FRACTION NUMBER, 22 ml/TUBE
0
0.25
0.50
0.75
1.00
1.25
1.50
1.75
0
1
2
3
4
5
6
7
0
20
40
60
80
100

Figure 22

Figure 23. Phosphocellulose II Chromatography of PP_i:F6P 1-phosphotransferase. A linear gradient of KCl was incorporated in the eluting buffer. All other details are given in the test.

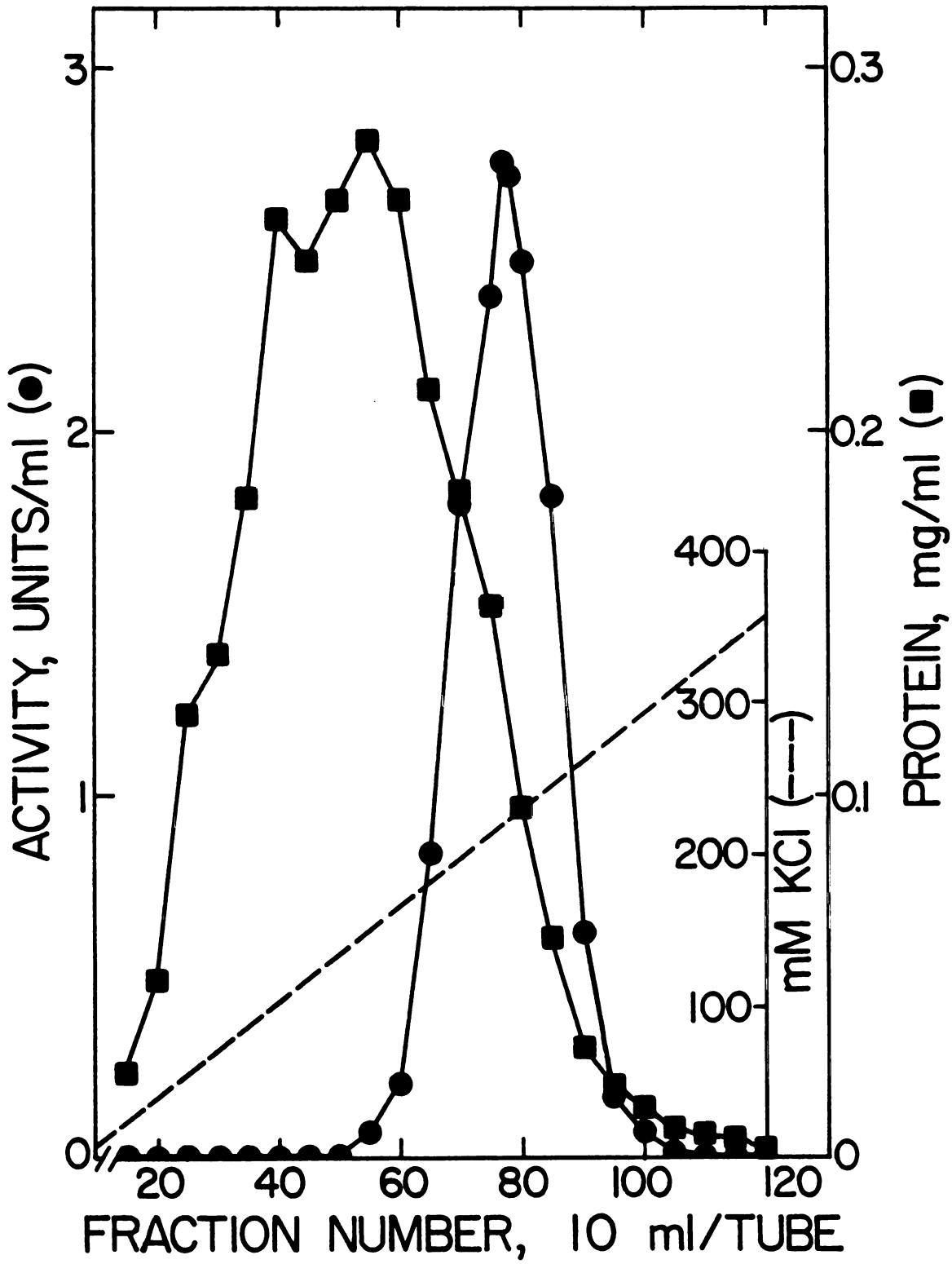
ACTIVITY, UNITS/ml (●)
PROTEIN, mg/ml (■)
mM KCl (---)
FRACTION NUMBER, 10 ml/TUBE
0
1
2
3
0.1
0.2
0.3
100
200
300
400
20
40
60
80
100
120

Figure 23

Figure 24. Bio-Gel A-1.5m chromatography of PP_i:F6P 1-phosphotransferase. All other details are given in the text.

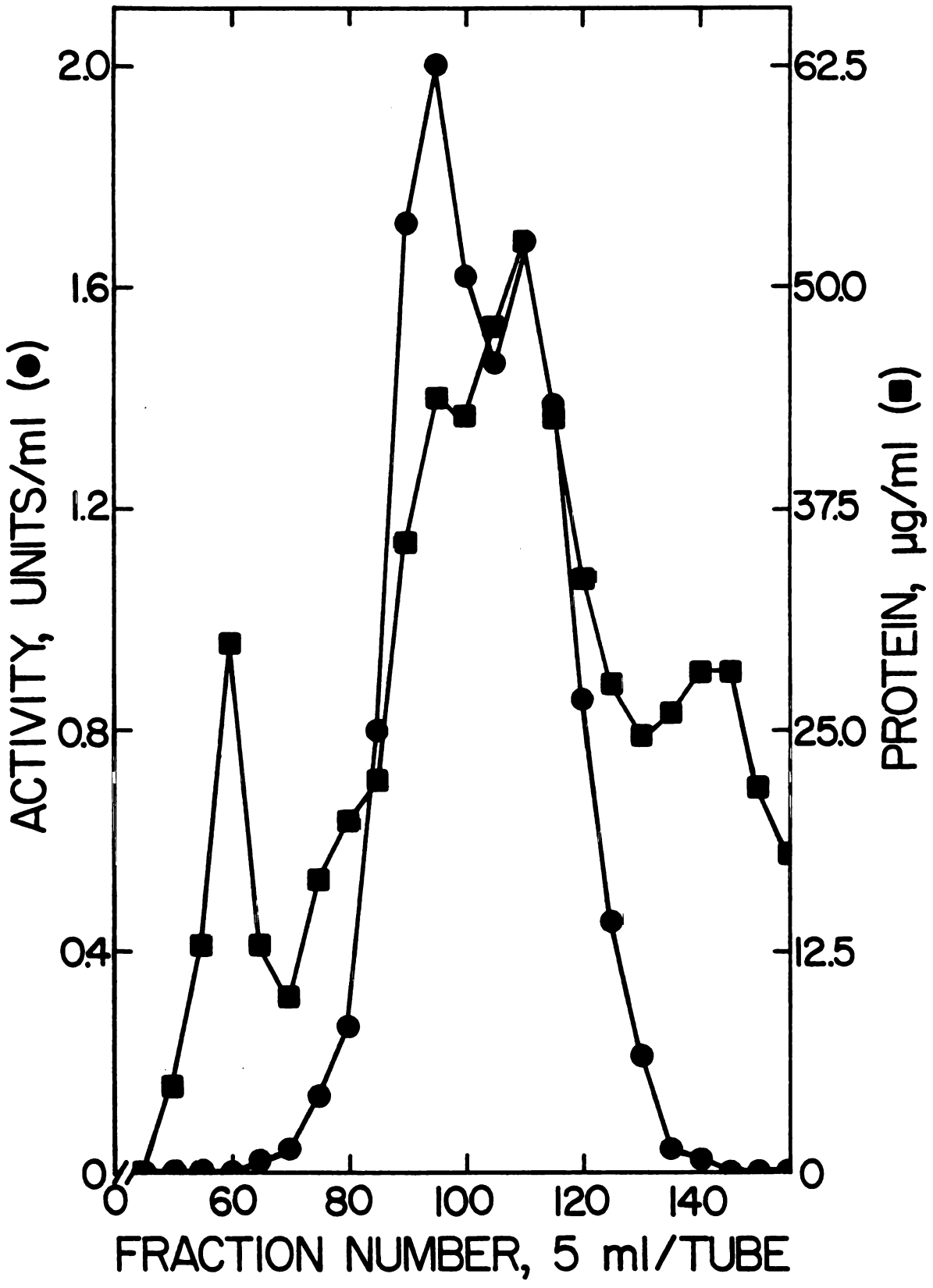
2.0
1.6
1.2
0.8
0.4
0
ACTIVITY, UNITS/ml (●)
62.5
50.0
37.5
25.0
12.5
0
PROTEIN, µg/ml (■)
0
60
80
100
120
140
FRACTION NUMBER, 5 ml/TUBE

Figure 24

enzyme in the pooled fractions from Bio-Gel A-1.5m step was 1030-fold purified with a 35% recovery. A summary of the purification is shown in Table 8.

Separation of two forms of the enzyme. As can be seen in Figure 24 or Figure 25a, the enzyme gave two peaks during the Bio-Gel A-1.5m step, indicating that there are two forms of the enzyme, a large form and a small form. Because the two peaks overlapped, the possibility of a dynamic equilibrium between the large and the small form existed, aside from the possibility that complete separation of the two forms may have been beyond the resolving capacity of the column. To determine if a dynamic equilibrium existed (151a), the pooled fraction from the first Bio-Gel A-1.5m step was rechromatographed on the same column at higher salt concentration; this also resulted in two peaks (Figure 25b). The fractions corresponding to peak I (Figure 25b, fractions 80-102) were pooled and rechromatographed on the same Bio-Gel A-1.5m column. Likewise, fractions corresponding to peak II (Figure 25b, fractions 103-125) were also pooled and rechromatographed separately on the same Bio-Gel A-1.5m column. The results are shown in Figure 25c. Peak I did not generate peak II, and peak II did not generate peak I, indicating that, indeed, they represent distinct forms of the enzyme which do not interconvert in an equilibrium situation. The previous purifications gave only one peak during the Bio-Gel A-1.5m step, and corresponded only to the small form. The main difference was that the earlier preparations had DTT included in the buffer and no glycerol was added. Since these results indicated that the enzyme exists in two forms (large and small), further experiments were conducted to investigate their properties.

Table 8. Purification of PP_i:F6P 1-Phosphotransferase from 500 g of mung beans.

	Fraction	Volume	Total protein[a]	Total activity	Specific activity	Recovery
		(mg)	(mg)	(units[b])	(units/mg protein)	(%)
1.	Crude extract	2030	26800	1020	0.038	(100)
2.	$(NH_4)_2SO_4$ ppt.	146	8000	1140	0.143	111
3.	DEAE-cellulose	77.0	1330	774	0.582	76
4.	Phosphocellulose I	77.0	201	543	2.70	53
5.	Phosphocellulose II	10.0	21.0	429	20.4	42
6.	Bio-Gel A-1.5m	10.0	9.20	359	39.0	35

[a]Protein was assayed by the procedure of Whitaker and Granum.

[b]One unit of activity is equivalent to 1 μmol per min of fructose 1,6-bisphosphate produced in the standard assay.

Figure 25. Separation of the two forms of PP_i:F6P 1-phosphotransferase by chromatography through Bio-Gel A-1.5m. The pooled fraction after phosphocellulose II chromatography (Figure 24) was chromatographed through a Bio-Gel A-1.5m column (3.4 x 95 cm) with a linear flow rate of 9.4 cm per hr. (a) In 10 mM Tris-acetate, pH 7.3 containing 10% glycerol, 0.1 mM Na EDTA and 20 mM KCl. (b) Rechromatography of the concentrated pooled fractions (80-125) from (a), with the KCl concentration in the buffer increased to 100 mM. (c) Rechromatography of the pooled fractions from (b) corresponding to peak I (fractions 80-102), and peak II (fractions 103-125), both in the same buffer as in run (b). Peaks I and II represent the large and small forms of the enzyme, respectively. All other details are given in the text.

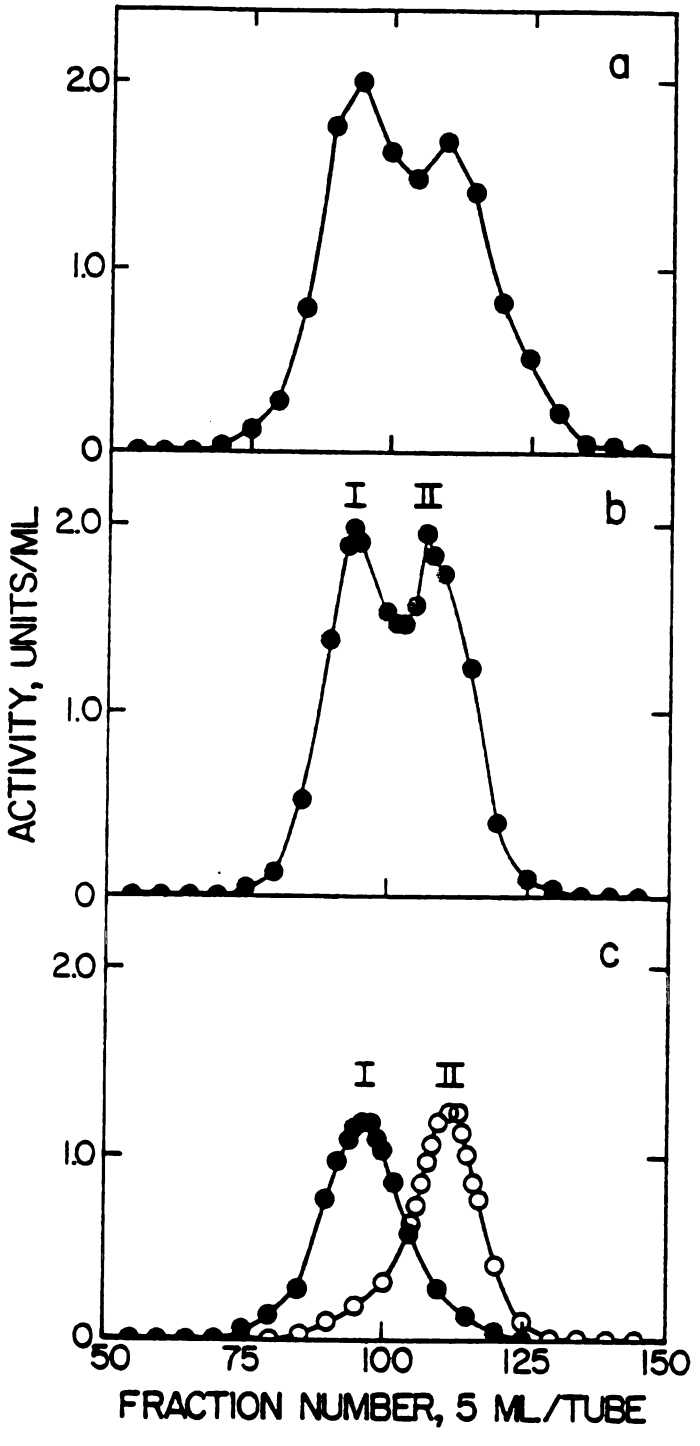
a
b
c
I
II
I
II
2.0
1.0
0
2.0
1.0
0
2.0
1.0
0
ACTIVITY, UNITS/ML
50
75
100
125
150
FRACTION NUMBER, 5 ML/TUBE

Figure 25

Some properties of the two forms. The enzyme preparations used for the determination of properties a through h below were from the third chromatographic run through Bio-Gel A-1.5m (Figure 25c), fraction number 90 (0.7 unit/ml, 1130-fold pure) representing the large form and fraction number 117 (0.7 unit/ml, 842-fold pure) representing the small form. The protein in fractions 90 and 117 was determined by the Bradford method (137).

a) Heat stability at 55°C. The small form was found to have a longer half-life ($t_{\frac{1}{2}}$ = 26 min) than the large form ($t_{\frac{1}{2}}$ = 14 min) as shown in Figure 26, indicating that the large form was more heat labile.

b) Effect of pH and buffer composition. The activity-pH response of both the large form (Figure 27) and the small form (Figure 28) were basically the same, having an optimum pH of 7.6-7.8 for both, whether the buffer used was Na-HEPES or Na-PIPES/Tris combination buffer. However, it may be noted that at pH 7.0-7.2 Na-PIPES buffer gave a higher activity that Na-HEPES for both forms. At pH 7.6-7.8 the Na-PIPES/Tris combination buffer was also slightly better than Na-HEPES buffer for both forms. Because the increase in the activity at pH 7.8 by using Na-PIPES/Tris combination buffer was only about 5% over the activity using Na-HEPES which was previously adopted as the buffer for the standard assay, no change in the buffer composition for the standard assay was made at this point. However, such information may be considered for future work on the enzyme.

c) K_A for fructose 2,6-bisphosphate at pH 7.8 and pH 7.0. Figure 29 shows that the large form had a higher affinity for fructose-2,6-P_2 (K_A = 25 nM, both at pH 7.8 and 7.0) than the small

Figure 26. Relative stability of the small and large forms of PP_i:F6P 1-phosphotransferase at 55°C. Two hundred microliters of each of the enzymes (large form, 0.70 unit/ml with a specific activity of 43 units/mg; and small form, 0.70 unit/ml with a specific activity of 32 units/mg) in 10 mM Tris-acetate buffer (pH 7.3, 100 mM KCl, 10% glycerol and 0.1 mM EDTA) was incubated in a water bath at 55°C. At each incubation period indicated, a 20 µl sample was transferred to a 1-ml tube, cooled in an ice bath for 1 min and 5 µl sample was assayed for its activity by standard assay (Method A).

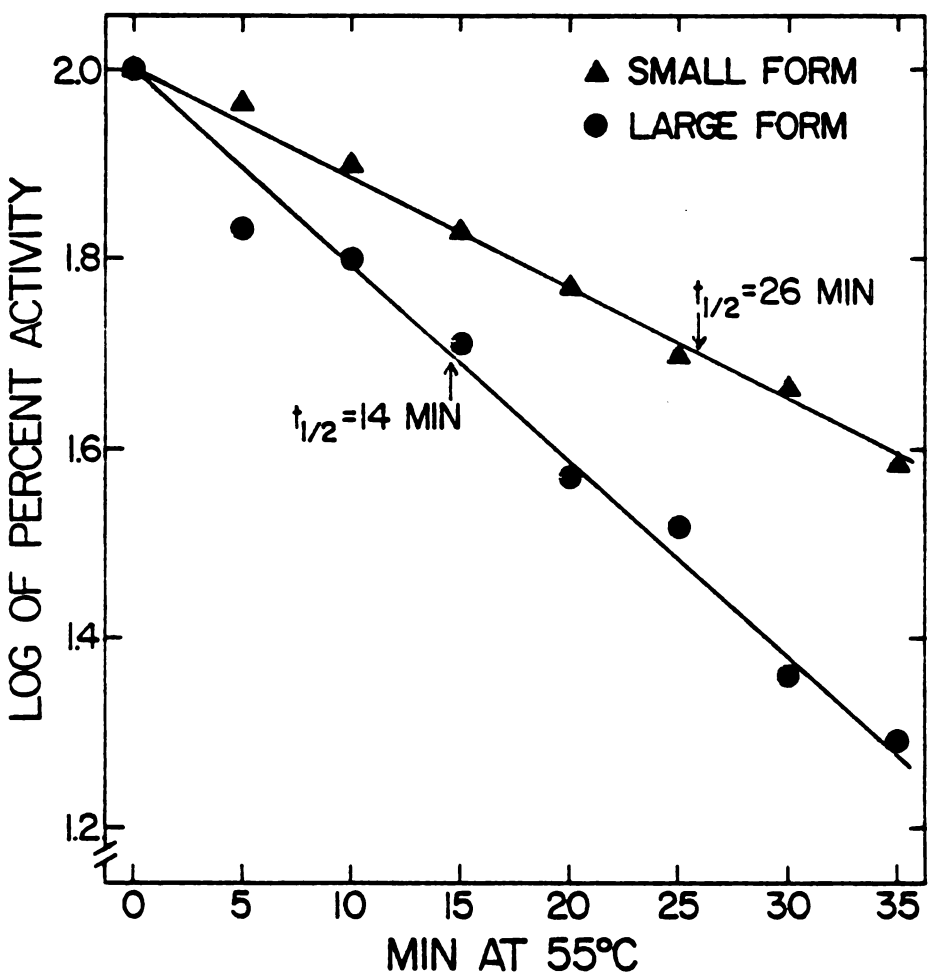
SMALL FORM
LARGE FORM
$t_{1/2}$=26 MIN
$t_{1/2}$=14 MIN
LOG OF PERCENT ACTIVITY
2.0
1.8
1.6
1.4
1.2
0
5
10
15
20
25
30
35
MIN AT 55°C

Figure 26

Figure 27. Effect of pH and buffer composition on the activity of the large form of PP_i:F6P 1-phosphotransferase. The reaction mixture (170 µl) contained 1.0 mM PP_i, 10 mM fructose-6-P, 1.0 mM Na EDTA, 6.0 mM $MgCl_2$, 0.24 mM Na NADH, 1.0 µM fructose-2,6-P_2, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase and 5 µl (2.5 mU at standard assay) of PP_i:F6P 1-phosphotransferase. The buffer concentration was 80 mM in each case; when PIPES and Tris were used in combination, the concentrations were 40 mM of each.

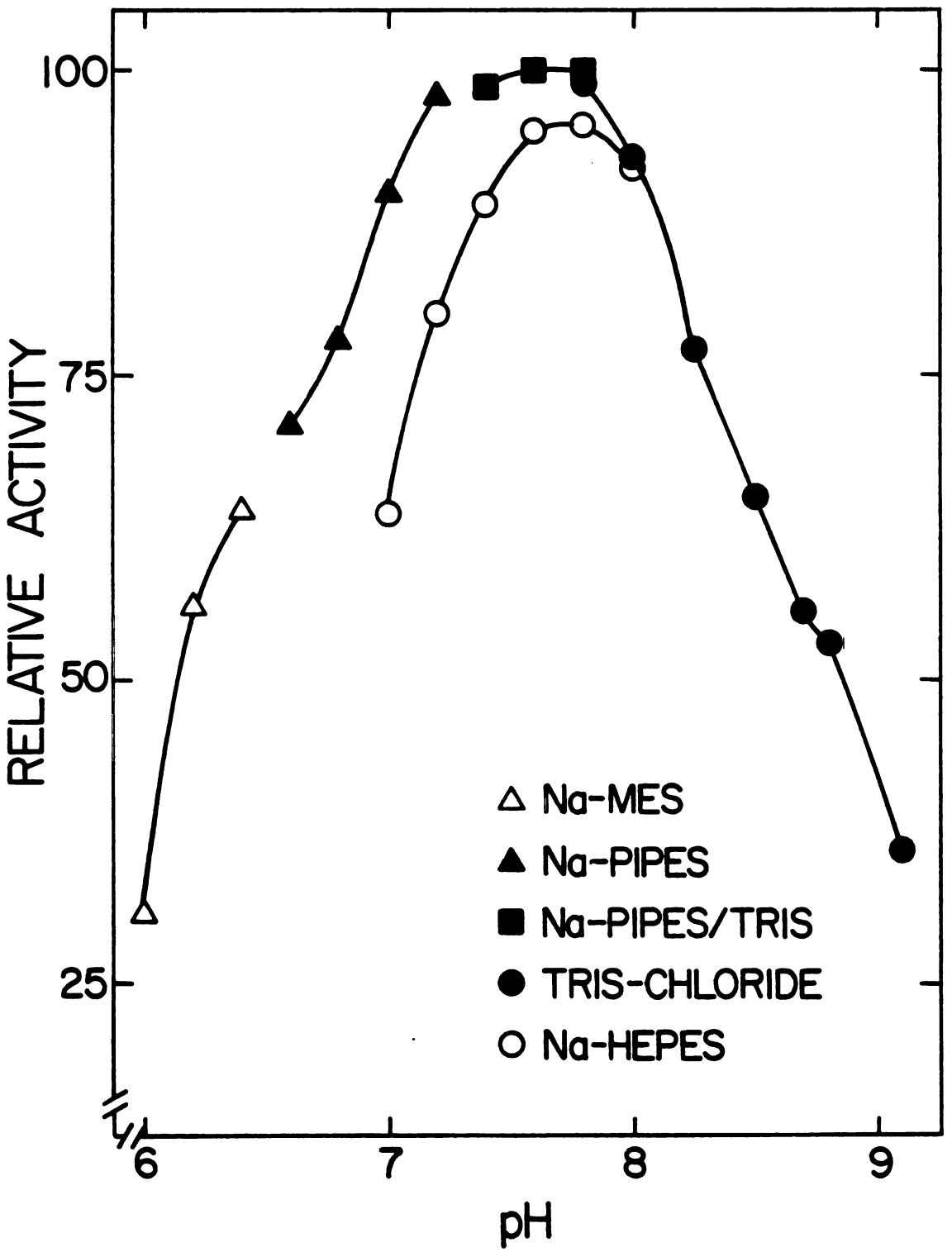
100
75
50
25
RELATIVE ACTIVITY
6
7
8
9
pH
Na-MES
Na-PIPES
Na-PIPES/TRIS
TRIS-CHLORIDE
Na-HEPES

Figure 27

Figure 28. Effect of pH and buffer composition on the activity of the small form of PP_i:F6P 1-phosphotransferase. The reaction mixture (170 µl) contained 1.0 mM PP_i, 10 mM fructose-6-P, 1.0 mM Na EDTA, 6.0 mM $MgCl_2$, 0.24 mM Na NADH, 1.0 µM fructose-2,6-P_2, 0.2 unit of fructose-1,6-P_2 aldolase, 2.0 units of triose-P isomerase, 0.2 unit of α-glycerol-P dehydrogenase and 5 µl (2.5 mU at standard assay) of PP_i:F6P 1-phosphotransferase. The buffer concentration was 80 mM in each case; when PIPES and Tris were used in combination, the concentrations were 40 mM of each.

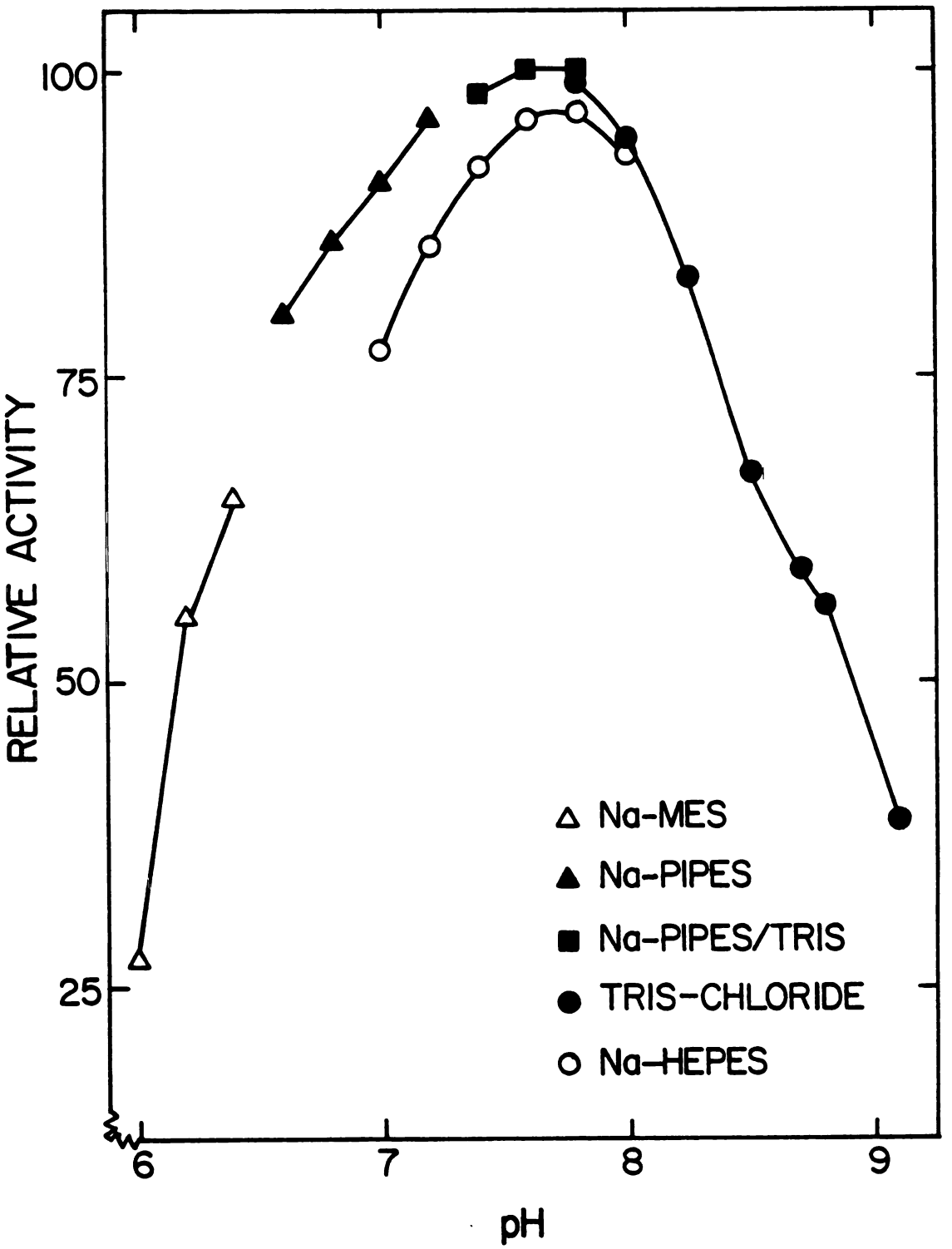
100
75
50
25
RELATIVE ACTIVITY
6
7
8
9
pH
Na-MES
Na-PIPES
Na-PIPES/TRIS
TRIS-CHLORIDE
Na-HEPES

Figure 28

Figure 29. Comparison of the large and small forms of PP_i:F6P 1-phosphotransferase with respect to the effect of pH on the K_A for fructose-2,6-P_2. The activity was determined by Method A assay, described in Materials and Methods. The velocity in this case is the increment over the velocities measured in the absence of fructose-2,6-P_2. The velocity is in terms of nmol fructose-2,6-P_2 formed per min by 5 µl of the enzyme preparation in each case.

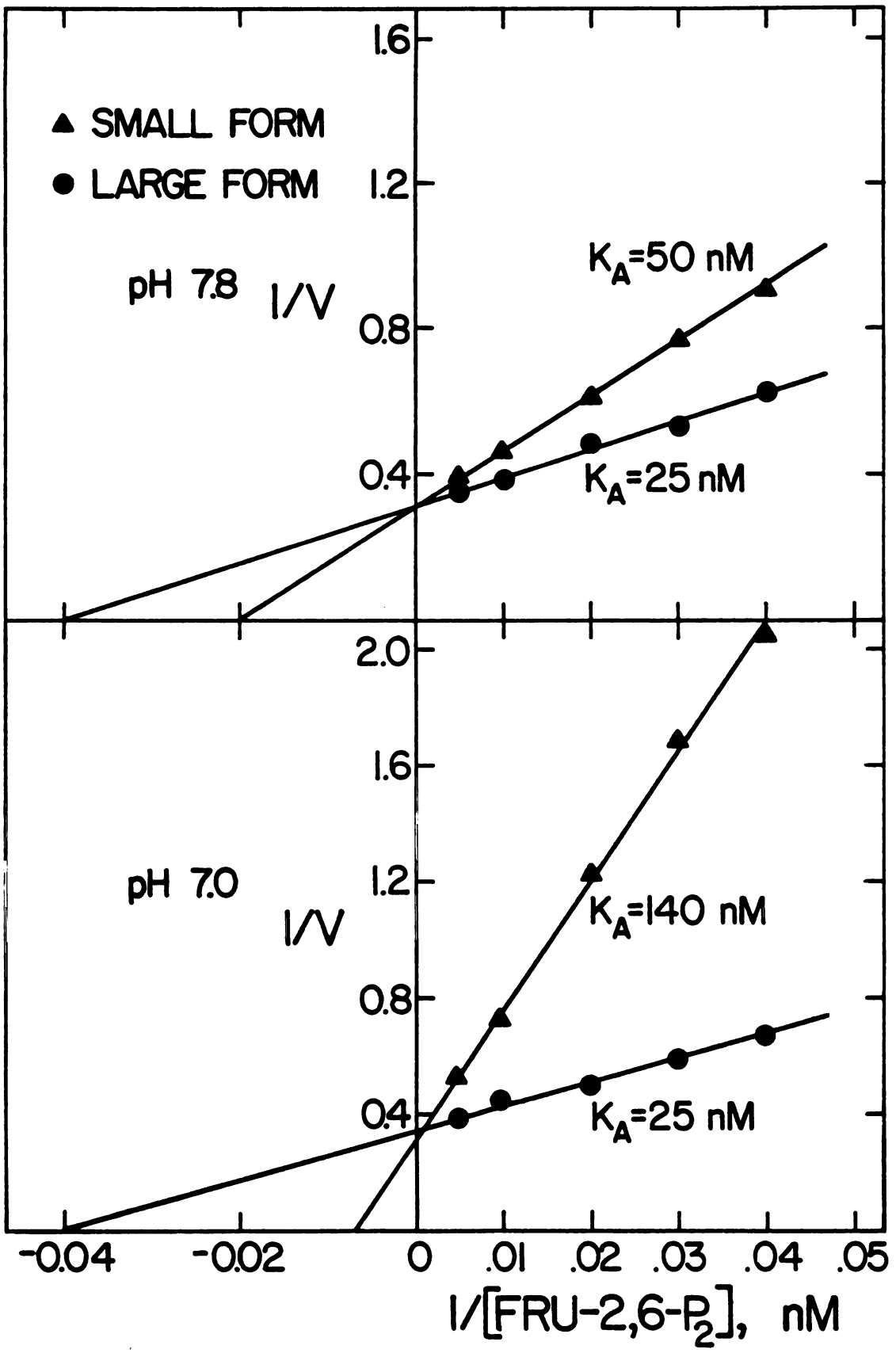
▲ SMALL FORM
● LARGE FORM
pH 7.8 1/V
K_A=50 nM
K_A=25 nM
pH 7.0 1/V
K_A=140 nM
K_A=25 nM
1.6
1.2
0.8
0.4
2.0
1.6
1.2
0.8
0.4
-0.04
-0.02
0
.01
.02
.03
.04
.05
1/[FRU-2,6-P_2], nM

Figure 29

form (K_A = 50 mM, at pH 7.8; and K_A = 140 mM at pH 7.0) suggesting that conversion of the small form to the large form may be a biologically significant means of activating the enzyme.

d) K_m for fructose 6-phosphate at pH 7.8 and pH 7.0. Figure 30 shows that the large and the small forms of the enzyme had the same affinity for fructose-6-P at pH 7.8 (K_m = 0.12 mM). At pH 7.0 there was a decrease in the affinity for the substrate for both forms, with even a greater decrease in affinity for the small form (K_m = 0.50 mM) than the large form (K_m = 0.15 mM). This again suggests that one means for the activation of the enzyme may be conversion from the small form to the large form.

e) K_m for PP_i at pH 7.8 and pH 7.0. The large and the small forms of the enzyme had the same affinity for PP_i at pH 7.8 (K_m = 0.09 mM) and at pH 7.0 (K_m = 0.13 mM); however, the affinity for PP_i was lower for both at pH 7.8 than at pH 7.0 (Figure 31).

f) K_m for fructose 1,6-bisphosphate. The large and small forms of the enzyme had the same affinity for fructose-1,6-P_2 at pH 7.8 (K_m = 0.07) (Figure 32).

g) K_m for P_i. The large and small forms of the enzyme had the same affinity for P_i at pH 7.8 (K_m = 0.28). It was also observed that the enzyme did not follow a truly hyperbolic kinetics at high concentration of P_i. Above 2 mM P_i the activity decreased (Figure 33).

h) Effect of P_i on the activity of the forward reaction. P_i which is a substrate for the reverse reaction inhibited the forward reaction. Figure 34 shows that in the absence of fructose-2,6-P_2, both

Figure 30. Comparison of the large and small forms of PP_i:F6P 1-phosphotransferase with respect to the effects of pH on the K_m for fructose-6-P. The activity was determined by Method A assay, described in Materials and Methods. The velocity is in terms of nmol fructose-1, 6-P_2 formed per min by 5 µl of the enzyme preparation in each case.

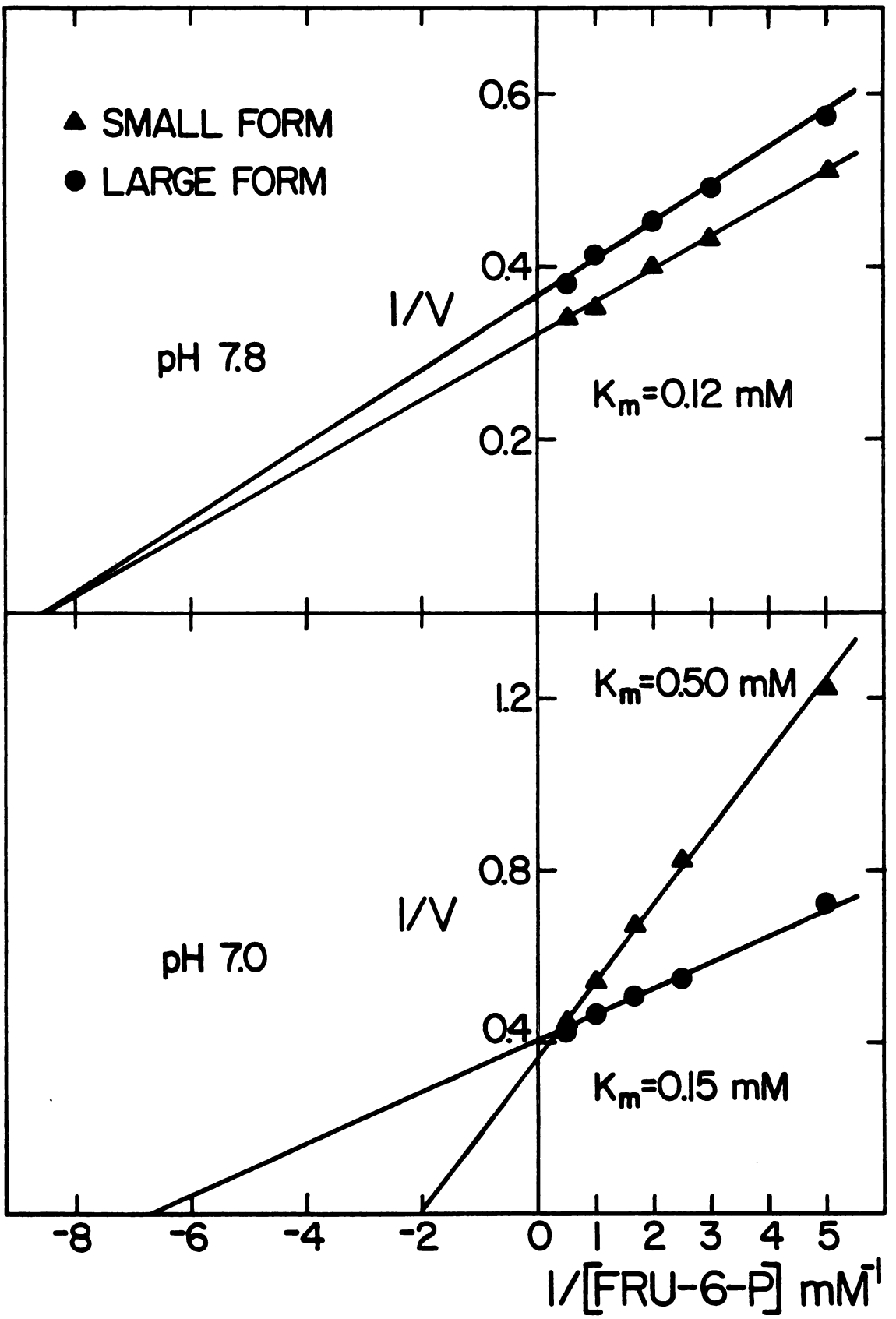
▲ SMALL FORM
● LARGE FORM
0.6
0.4
1/V
pH 7.8
K_m=0.12 mM
0.2
K_m=0.50 mM
1.2
0.8
1/V
pH 7.0
0.4
K_m=0.15 mM
-8
-6
-4
-2
0
1
2
3
4
5
1/[FRU-6-P] mM^{-1}

Figure 30

Figure 31. Comparison of the large and small forms of PP_i:F6P 1-phosphotransferase with respect to the effect of pH on the K_m for PP_i. The activity was determined by Method A assay, described in Materials and Methods. The velocity is in terms of nmol fructose-1, 6-P_2 formed per min by 5 µl of the enzyme preparation in each case.

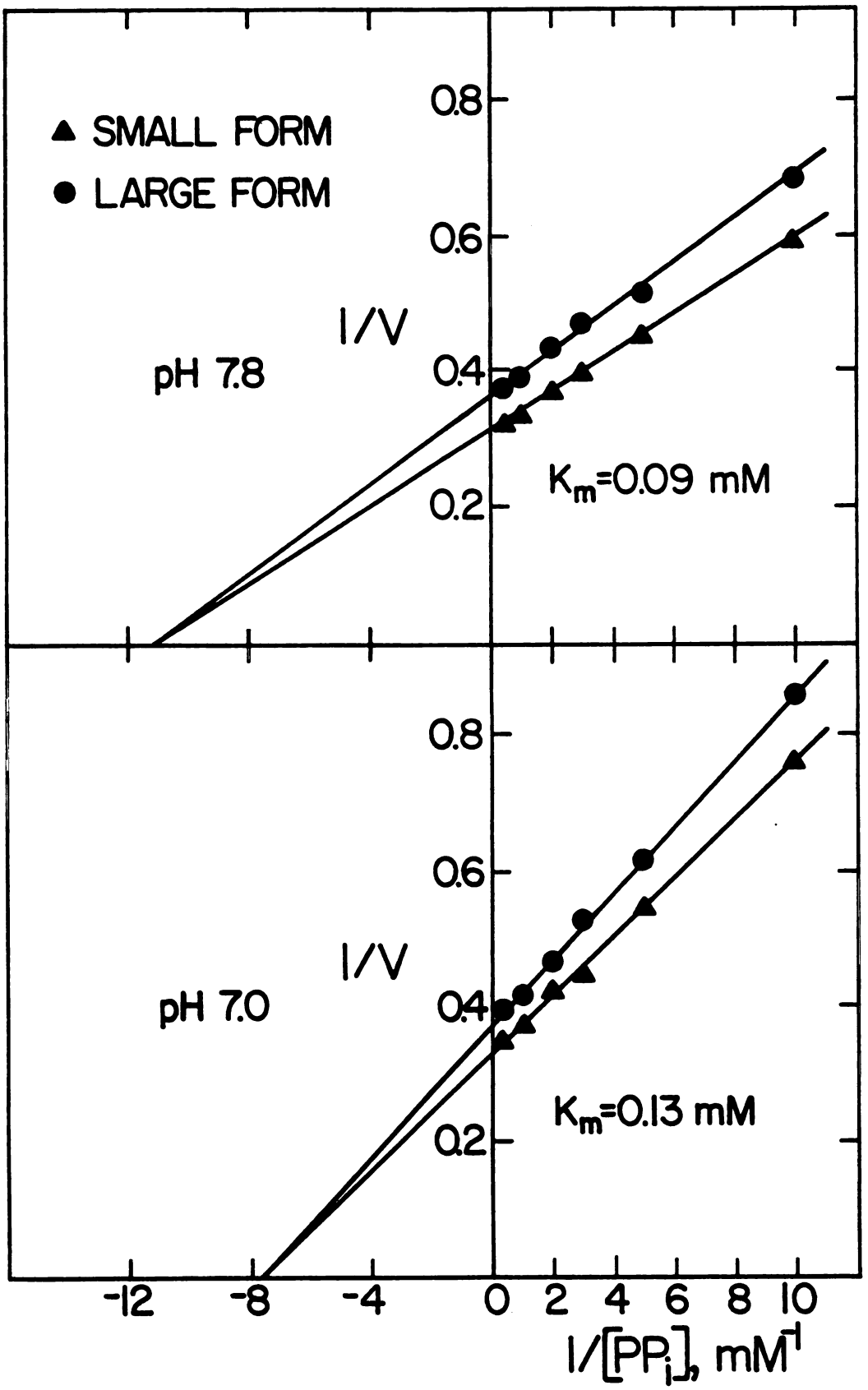
▲ SMALL FORM
● LARGE FORM
pH 7.8
1/V
0.8
0.6
0.4
0.2
K_m=0.09 mM
pH 7.0
1/V
0.8
0.6
0.4
0.2
K_m=0.13 mM
-12
-8
-4
0
2
4
6
8
10
1/[PP_i], mM^{-1}

Figure 31

Figure 32. Comparison of the large and small forms of PP_i:F6P 1-phosphotransferase with respect to the K_m for fructose-1,6-P_2 at pH 7.8. The activity was determined by Method C assay, described in Materials and Methods. The velocity is in nmol fructose-1,6-P_2 consumed per min by 10 µl of the enzyme preparation in each case.

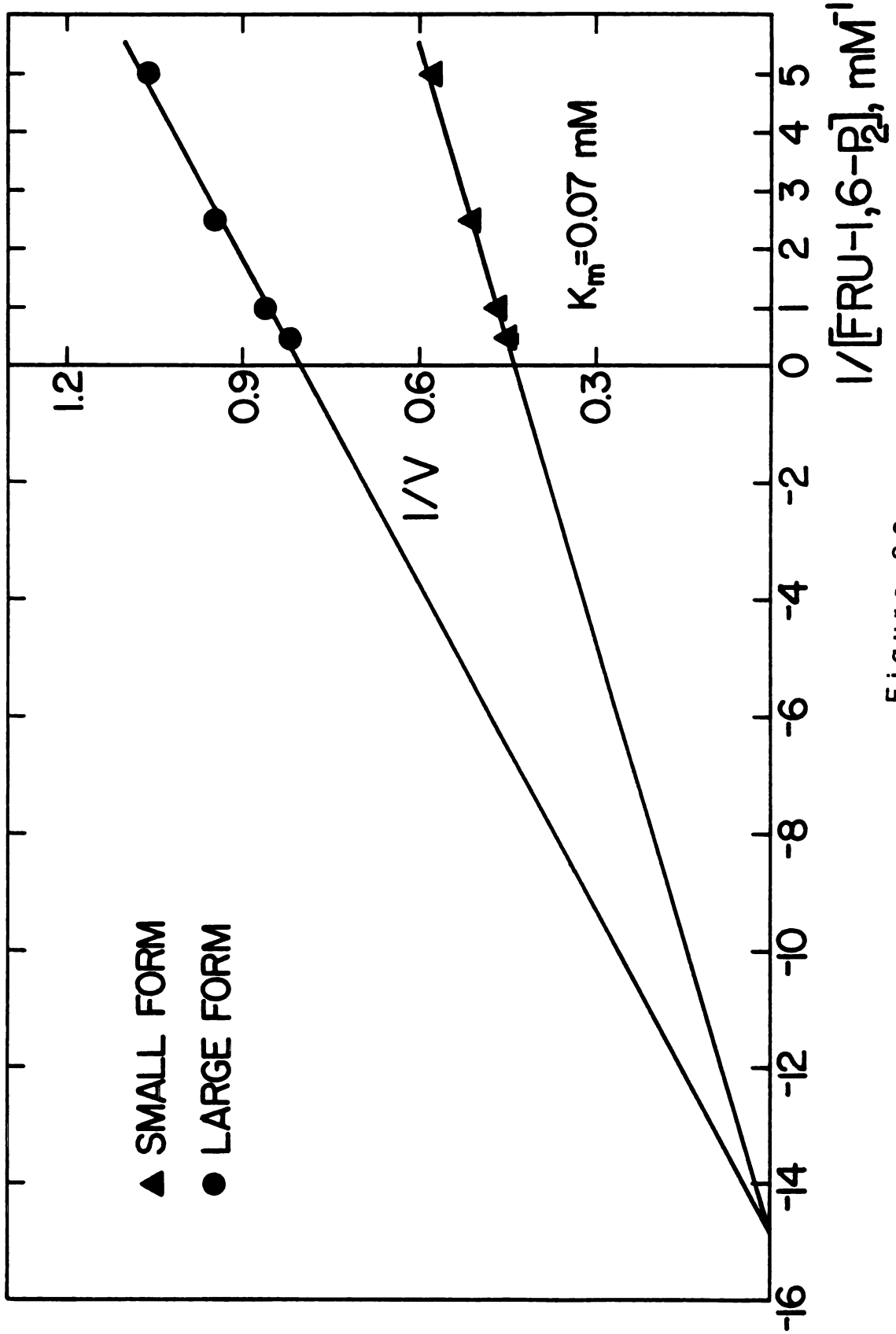
▲ SMALL FORM
● LARGE FORM
1/V
1.2
0.9
0.6
0.3
K_m=0.07 mM
0
-2
-4
-6
-8
-10
-12
-14
-16
1
2
3
4
5
1/[FRU-1,6-P_2], mM^{-1}

Figure 32

Figure 33. Comparison of the large and small forms of PP_i:F6P 1-phosphotransferase with respect to the K_m for P_i at pH 7.8. The activity was determined by Method C assay, described in Materials and Methods. The velocity is in nmol fructose-1,6-P_2 consumed per min by 10 µl of the enzyme preparation in each case.

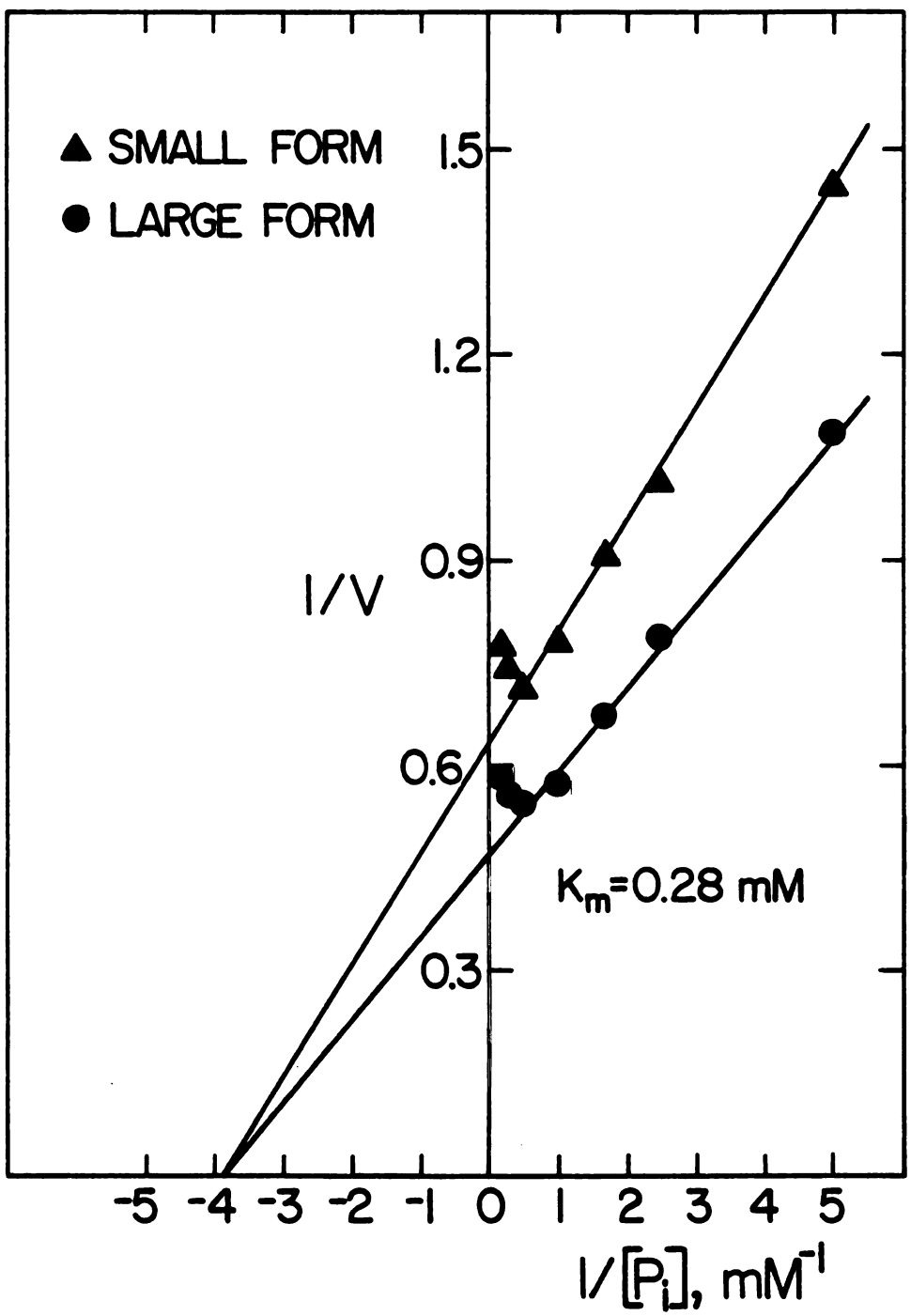
▲ SMALL FORM
● LARGE FORM
1.5
1.2
0.9
0.6
0.3
1/V
K_m=0.28 mM
-5 -4 -3 -2 -1 0 1 2 3 4 5
1/[Pi], mM^{-1}

Figure 33

Figure 34. Effect of P_i on the activity of the two forms of PP_i:F6P 1-phosphotransferase in the absence and presence of two concentrations of fructose-2,6-P_2. The activity was determined as in the standard assay, Method A (forward reaction), with modifications indicated.

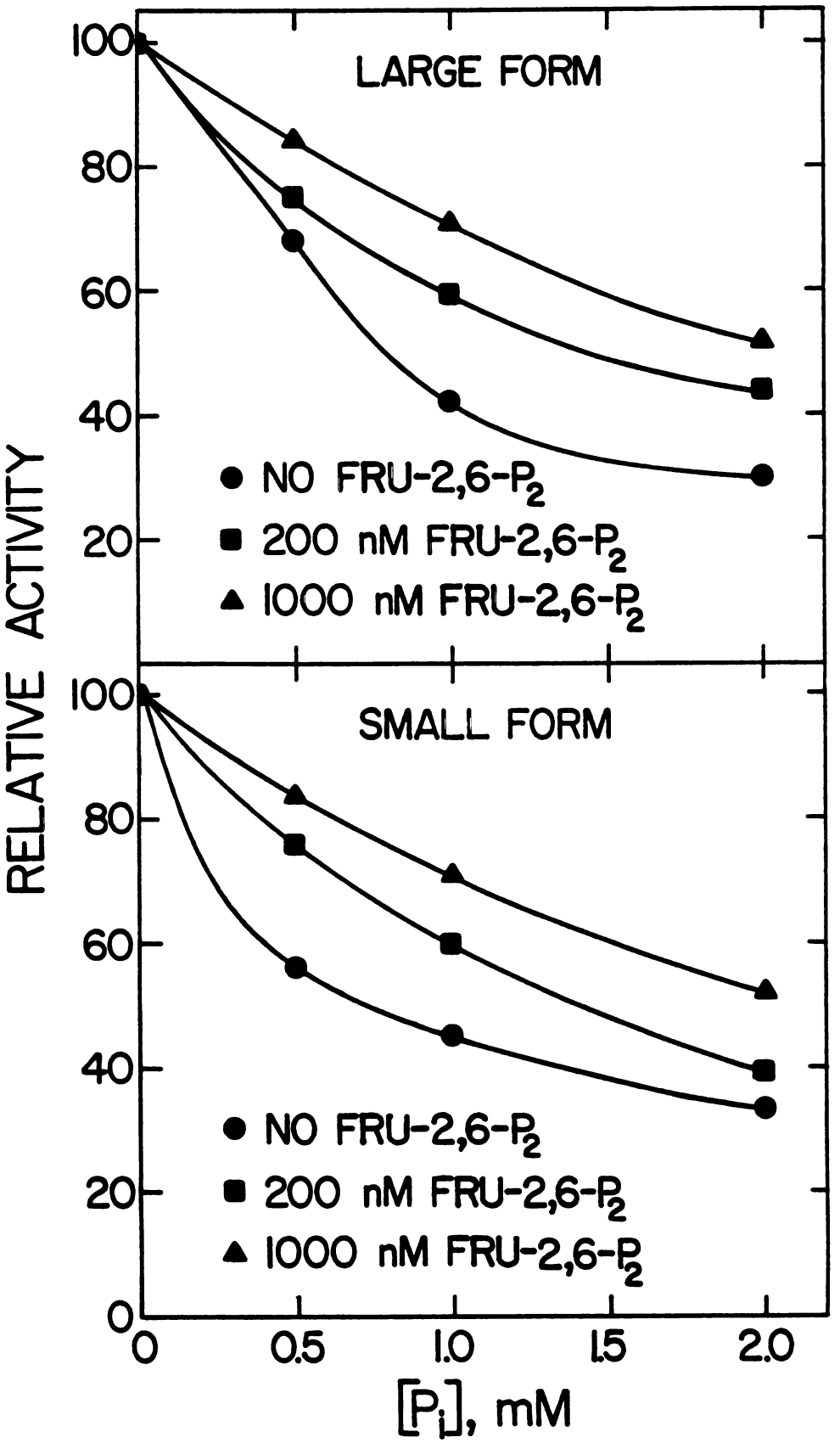
LARGE FORM
SMALL FORM
NO FRU-2,6-P2
200 nM FRU-2,6-P2
1000 nM FRU-2,6-P2
RELATIVE ACTIVITY
[Pi], mM
0
20
40
60
80
100
0.5
1.0
1.5
2.0

Figure 34

the large and small forms of the enzyme were severely inhibited by P_i (large form, 50% inhibition at 0.78 mM P_i; and small form, 50% inhibition at 0.70 mM P_i). In the presence of 200 nM fructose-2,6-P_2, the degree of inhibition by P_i was less severe for both the large and the small forms (large form, 50% inhibition at 1.90 mM P_i; and small form, 50% inhibition at 1.95 mM P_i). At saturating amounts of fructose-2,6-P_2 (1000 nM), the P_i concentration that was necessary to inhibit by 50% was greater than 2 mM for both the large and the small forms of the enzyme.

i) Molecular weight by gel-filtration on Bio-Gel A-1.5m. Determination of the molecular weights of the two forms of the enzyme by column chromatography yielded values of 170,000 for the small form and 340,000 for the large form (Figures 35 and 36).

Apparent conversion of the large form to the small form upon incubation with DTT. During previous purification procedures, when DTT was included as one of the components of the buffers, only the small form of the enzyme was detected, suggesting that DTT may be responsible for the conversion of the large form to the small form. In addition, the two forms of the enzyme had a large difference in their K_A values for fructose-2,6-P_2 at pH 7.0 (large form, K_A = 25 nM; and small form, K_A = 140 nM). From these observations, it should be possible to obtain a presumptive evidence for the transformation of the large form to the small form by monitoring the K_A after incubation with DTT. Figure 37 shows an apparent DTT-dependent change in K_A for fructose-2,6-P_2 as reflected by a change in the activation ratio of the large form to that of the small form, suggesting a concomitant change from the large form to the small form. The "activation ratio"

Figure 35. Elution profile of molecular weight standards and the two forms of PP_i:F6P 1-phosphotransferase chromatographed on Bio-Gel A-1.5m. A 5.6-ml sample containing 40 units of rabbit muscle pyruvate kinase, 20 units of rabbit muscle aldolase, 40 units of yeast hexokinase and about 10 units each of the two forms of PP_i:F6P 1-phosphotransferase were chromatographed using a column (3.4 x 94.5 cm) of Bio-Gel A-1.5m equilibrated with a buffer (10 mM Tris-acetate, pH 7.3, containing 100 mM KCl, 10% glycerol and 0.1 mM Na EDTA). Elution of the proteins was done using the same buffer, collecting 5.0-ml fractions at a flow rate of 80 ml per hr. A separate run, on the same column, of 15 mg horse spleen ferritin and 40 units of yeast hexokinase was conducted to determine the elution profile of ferritin, the concentration of which was determined by its absorbance at 280 nm. The yeast hexokinase served a a countercheck for any possible variation of the separate run. Enzymatic assays are described in the text. Symbols: □ horse spleen ferritin, ● PP_i:F6P 1-phosphotransferase, ▲ rabbit muscle pyruvate kinase, ■ rabbit muscle aldolase, and ○ yeast hexokinase.

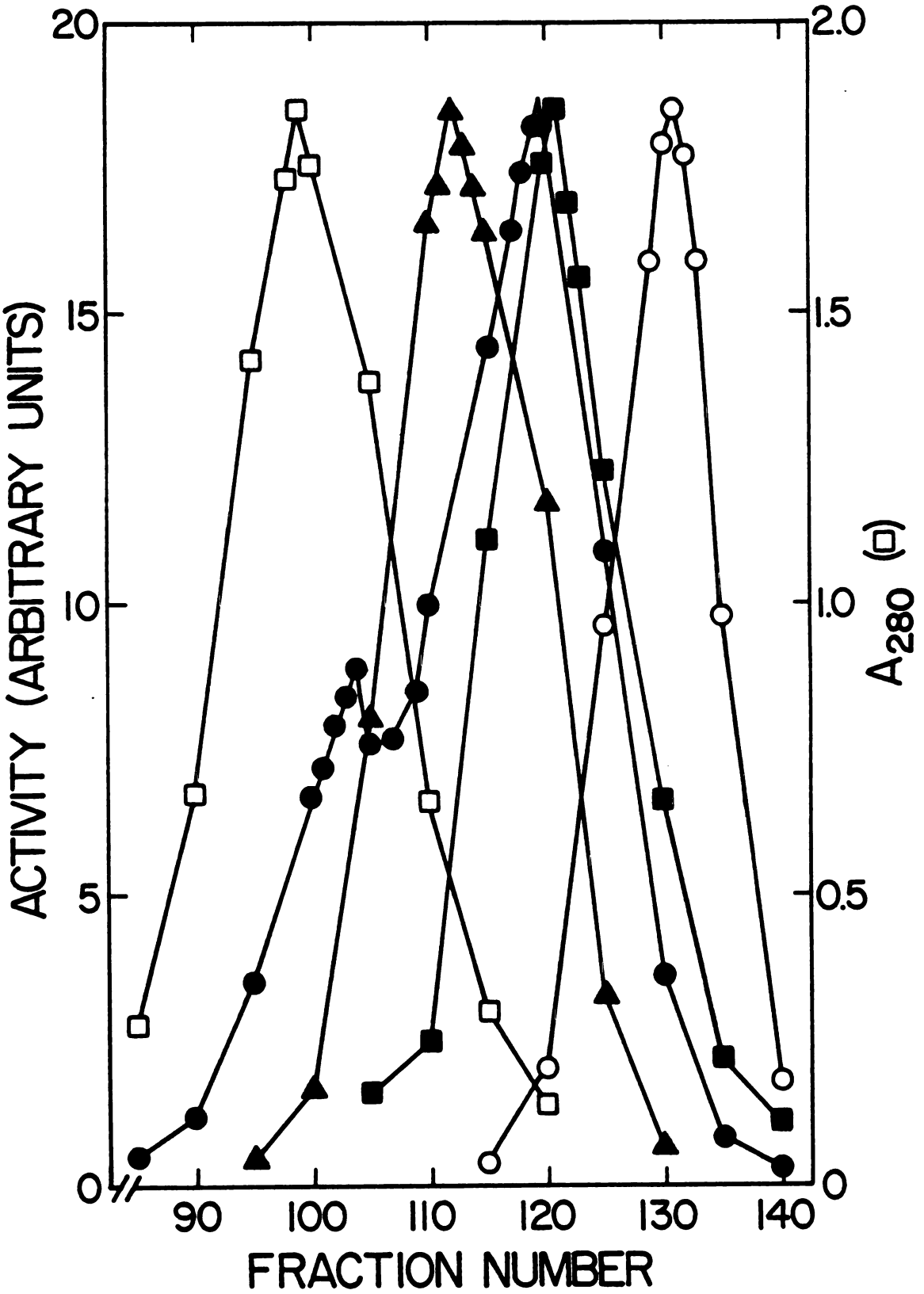
20
15
10
5
0
2.0
1.5
1.0
0.5
0
90
100
110
120
130
140
ACTIVITY (ARBITRARY UNITS)
A280 (□)
FRACTION NUMBER

Figure 35

Figure 36. Semi-log plot of the molecular weight of the standard proteins (horse spleen ferritin, M_r = 450,000; rabbit muscle pyruvate kinase, M_r = 237,000; rabbit muscle aldolase, M_r = 160,000, and yeast hexokinase, M_r = 102,000) versus the peak fraction number from the elution profile on the Bio-Gel A-1.5m column (Figure 35). The molecular weights of the large and the small forms of PP_i:F6P 1-phosphotransferase were interpolated from their peak fractions.

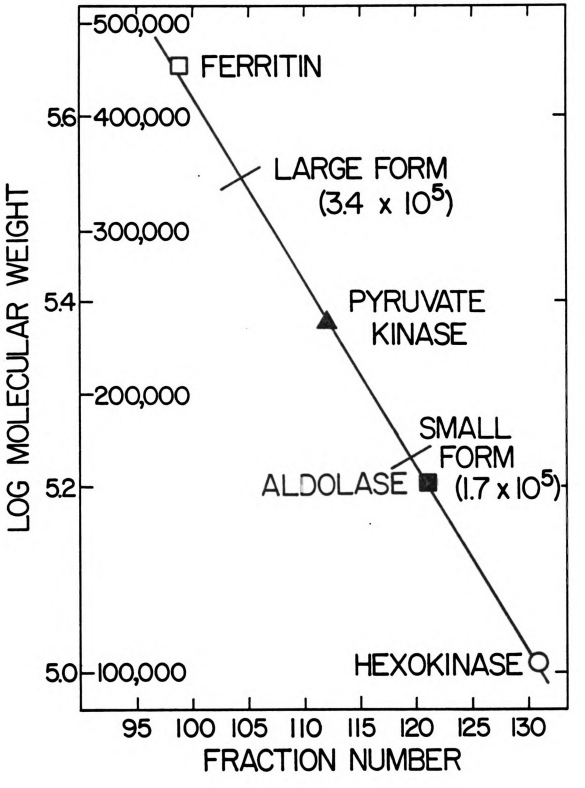
500,000
FERRITIN
5.6
400,000
LARGE FORM
(3.4 x 10^5)
300,000
PYRUVATE
KINASE
5.4
200,000
SMALL
FORM
ALDOLASE
(1.7 x 10^5)
5.2
5.0
100,000
HEXOKINASE
LOG MOLECULAR WEIGHT
95 100 105 110 115 120 125 130
FRACTION NUMBER

Figure 36

Figure 37. Apparent DTT-dependent change in K_A for fructose-2,6-P_2 for the large form of PP_i:F6P 1-phosphotransferase, suggesting a concomitant change to the small form of the enzyme. Aliquots of pooled fractions representing the large and small forms of the enzyme were incubated in 15 mM DTT. The activity was determined as in the standard assay (at 0, 35 and 1000 nM fructose-2,6-P_2) after timed periods of incubation. In each assay, 2.0 µl (original activity of 2.5 mU) of the large and small forms were used giving a final concentration of 0.18 mM DTT in the assay mixture. The "activation ratio" is the fold activation when assayed in the absence and presence of 35 nM fructose-2,6-P_2 divided by the fold activation when assayed in the absence and presence of 1000 nM fructose-2,6-P_2, and therefore is a reflection of K_A.

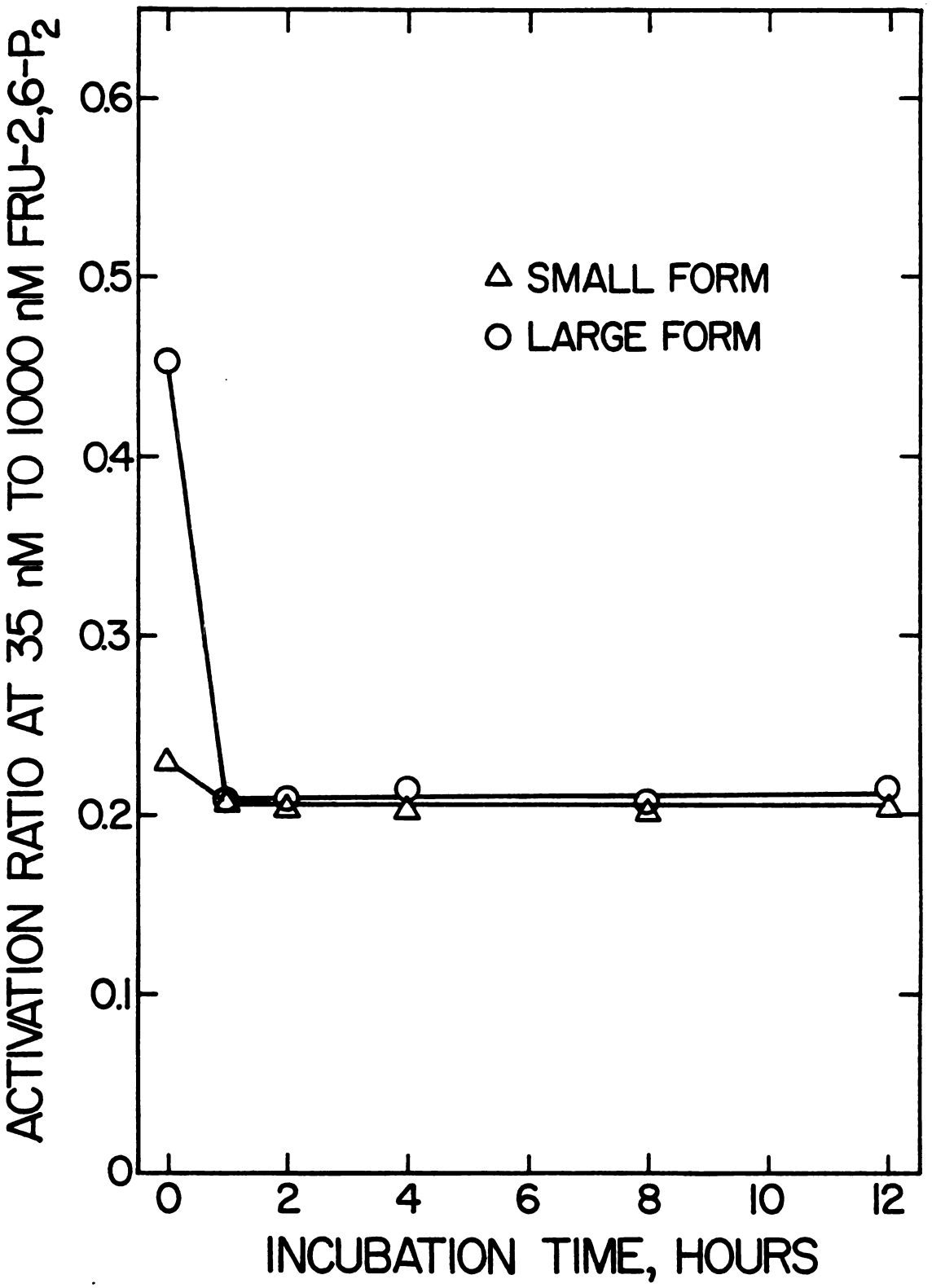
ACTIVATION RATIO AT 35 nM TO 1000 nM FRU-2,6-P2
0.6
0.5
0.4
0.3
0.2
0.1
0
△ SMALL FORM
○ LARGE FORM
0
2
4
6
8
10
12
INCUBATION TIME, HOURS

Figure 37

is the fold activation when assayed in the absence and presence of 35 nM fructose-2,6-P_2 divided by the fold activation when assayed in the absence and presence of 1000 nM fructose-2,6-P_2, and therefore is a reflection of K_A. At zero time of incubation, the activation ratio for the large and small forms were 0.46 and 0.23, respectively, which were consistent with what is predicted by a low K_A for the large form and higher K_A for the small form. Upon incubation with DTT, from 1 hour on, the activation ratio for both the large and small forms were essentially equal, about 0.21, suggesting that the large form may have been transformed to the small form by the action of DTT. The 0.21 activation ratio was about equal to the theoretical value of the activation ratio that would be predicted by Michaelis-Menten equation for the small form as follows:

$$\text{Activation ratio} = \frac{(V \text{ at } 35 \text{ nM}) - (V \text{ at zero nM})}{(V \text{ at } 1000 \text{ nM}) - (V \text{ at zero nM})}$$

$$= \frac{V_m\,(S_1)/[K_A + (S_1)]}{V_m\,(S_2)/[K_A + (S_2)]}$$

$$= 0.228 \text{ (at } V_{max} = 1,\ K_A = 140 \text{ nM},\ S_1 = 35 \text{ nM and } S_2 = 1000 \text{ nM)}$$

Survey of PP_i:F6P 1-Phosphotransferase and Fructose 2,6-Bisphosphate in Some Plant Species.

A survey of the occurrence of the enzyme in plants indicated that the enzyme is widespread among plant species (Table 9). The enzyme could also be found in various plant parts: seeds, leaves, bulb, roots, stalks and fruits.

Table 9. Occurrence of PP_i:F6P 1-Phosphotransferase in Some Plant Species

SPECIES	PART	SPECIFIC ACTIVITY[a]
		(mU/mg protein)[b]
MUNG BEANS *Phaseolus aureus*	GERMINATED SEED	36.0
SOYBEAN, *Glycine max*	GERMINATED SEED	9.1
PEAS, *Pisum sativa*	GERMINATED SEED	5.2
SPINACH, *Spinacia oleracea*	LEAVES	7.3
CORN, *Zea mays*	GERMINATED SEED	8.4
SCALLION, (GREEN ONIONS) *Allium schoenoprasum*	BULB	30.0
	ROOTS	1.5
	LEAVES	4.9
CARROT, *Daucus carota*	ROOTS	0.3
ONION, *Allium cepa*	BULBS	4.7
CELERY, *Apium graveolens*	STALKS	6.5
	LEAVES	1.4
GREEN PEPPER, *Capsicum annuum*	FRUIT	13.0
WALNUTS, *Juglans* sp.	SEED	22.0
BANANA, *Musa acuminata*	RIPE FRUIT	0.1
DUCKWEED, *Lemna minor*	WHOLE PLANT	8.2

[a]PPi:F6P 1-Phosphotransferase activity was assayed by method A (forward reaction, standard assay).

[b]Protein was determned by the method of Whitaker and Granum (134).

Fructose-2,6-P_2 was also found in several plant species (Table 10). It is present in seeds, leaves, bulb, roots and fruits.

Table 10. Occurrence of Fructose 2,6-Bisphosphate in Some Plant Species. PP_i:F6P 1-Phosphotransferase was used as a reagent for determining the presence of fructose-2,6-P_2. The assay was basically Method A (forward reaction, standard assay) except for the omission of fructose-2,6-P_2. The response of the enzyme to activation by column fractions of extracts was the basis for determining the presence of fructose-2,6-P_2.

SPECIES	PART	RELATIVE AMOUNTS
MUNG BEANS *Phaseolus aureus*	GERMINATED SEED	++
	DRY SEED	+
PEAS, *Pisum sativa*	GERMINATED SEED	++
ONION, *Allium cepa*	BULB	++
SCALLION, (GREEN ONIONS) *Allium schoenoprasum*	BULB	+
	ROOTS	++
	LEAVES	++
CARROT, *Daucus carota*	LEAVES	++
BANANA, *Musa acuminata*	RIPE FRUIT	++
SPINACH, *Spinacia oleracea*	LEAVES	++

++ Significant amount

+ Trace amount

DISCUSSION

The detection of PP_i:F6P 1-phosphotransferase activity in mung beans establishes the presence of the enzyme in C_3 plants. Prior to the report by Sabularse and Anderson (138), the enzyme had only been found in a few microbes and in leaves of pineapple, a crassulacean plant (21). In addition, no regulatory mechanism for the enzyme from any source, namely its activation by hexose bisphosphates, has been previously reported.

The activation of the enzyme by fructose-1,6-P_2 (a product of the reaction it catalyzes) was established indirectly by a series of experiments, and directly by following the rate of P_i formation using a glyceraldehyde-3-P dehydrogenase-coupled assay wherein the response of the enzyme to activation by the accumulated fructose-1,6-P_2 during the reaction was demonstrated. It was also found that millimolar amounts of glucose-1,6-P_2 could substitute for micromolar amounts of fructose-1,6-P_2 to the same degree of activation. For a time, the aldolase-coupled assay with glucose-1,6-P_2 as an activator was conveniently employed to assay for the enzyme even in the crude extract.

Fructose-1,6-P_2 and glucose-1,6-P_2 are known activators of ATP-dependent phosphofructokinase from animal sources and some microorganisms (148, 151b, 151c). During the progress of this thesis research, however, fructose-2,6-P_2 was reported to be a naturally

occurring activator for ATP-dependent phosphofructokinase in animal tissues (see Review of Literature section). When fructose-2,6-P_2 was tried as a possible activator for PP_i:F6P 1-phosphotransferase, it was found that nanomolar amounts (K_A = 50 nM) could replace micromolar amounts of fructose-1,6-P_2 (K_A = 17 μM) or millimolar amounts of glucose-1,6-P_2 (K_A = 0.4 mM) to give the same or even greater degree of activation. Fructose-2,6-P_2 was also demonstrated in this investigation to be a naturally occurring compound in mung bean (139) and other plants, in addition to being the most potent activator of PP_i:F6P 1-phosphotransferase, suggesting that it is the physiologically significant activator for the enzyme. Because the enzyme activity is inhibited by P_i even in the presence of fructose-2,6-P_2, the V_{max} for the reverse reaction is lower than that of the forward reaction, favoring a glycolytic role for the enzyme. In E. histolytica and P. shermanii, the PP_i:F6P 1-phosphotransferase appeared to play a major role in glycolytic pathway (17, 18). However, when P. shermanii was grown on lactate or glycerol, the activity of the PP_i-dependent phosphofructokinase was 15-20 times that of the fructose bisphosphatase, which led to the speculation that the enzyme may function in a gluconeogenic as well as glycolytic capacity (18). E. histolytica apparently contains no fructose bisphosphatase (2), so that the enzyme in this amoeba may have both a glycolytic and a gluconeogenic role. The enzyme in microorganisms apparently does not require an activator.

PP_i is generated in many biochemical processes (see Introduction). In germinating starchy seeds, such as mung bean, a major source of PP_i would be the conversion of starch to sucrose:

one mole of PP_i is formed by UDP-glucose pyrophosphorylase for each mole of sucrose synthesized. Because sucrose is the main transport form of sugars (114), considerable PP_i would be generated in sucrose synthesis alone. In addition, inorganic pyrophosphatase activity in germinating mung bean seeds is undetectable (Table 4), suggesting that mung bean seeds grown in the dark would contain levels of PP_i sufficiently high to serve as a phosphoryl donor. In support of this position, Simmons and Butler (146) and Bucke (147) reported that the intracellular localization of inorganic pyrophosphatase in the leaves of higher plants is in the chloroplast. Furthermore, Simmons and Butler (146) reported that mature seeds of maize have no pyrophosphatase.

The observed effect of P_i, AMP, citrate and ADP on the activity of the ATP-dependent phosphofructokinase in mung bean is in agreement with what is generally reported for the enzyme in other plants (148). ADP and AMP are both positive effectors for mammalian ATP-dependent phosphofructokinase, but are negative effectors for the plant enzyme (148). The potent inhibitory effects of phosphoenolpyruvate observed for the mung bean ATP-dependent phosphofructokinase has also been observed for the pea seed enzyme by Kelly and Turner (150).

It is interesting to note that P_i, which activates the ATP-dependent phosphofructokinase, inhibits PP_i:F6P 1-phosphotransferase. Likewise, phosphoenolpyruvate, which inhibits the ATP-dependent phosphofructokinase at micromolar levels, does not affect the activity of PP_i:F6P 1-phosphotransferase even at 100 times the concentration that inhibits the ATP-dependent phosphofructokinase. ATP at 1 mM is non-inhibitory for the ATP-dependent phosphofructokinase but is

slightly inhibitory to PP_i:F6P 1-phosphotransferase. Conversely, 2 mM PP_i, which is not inhibitory to PP_i:F6P 1-phosphotransferase, is inhibitory for ATP-dependent phosphofructokinase. ADP, AMP, and citrate inhibit the ATP-dependent phosphofructokinase but do not affect PP_i:F6P 1-phosphotransferase activity. Furthermore, PP_i:F6P 1-phosphotransferase is activated by nanomolar amounts of fructose-2, 6-P_2, whereas no effect is observed for the ATP-dependent phosphofructokinase even at substrate concentrations that are below the K_m.

PP_i:F6P 1-phosphotransferase exhibited Michaelis-Menten kinetics both in the absence and presence of an activator. Fructose-2,6-P_2 (1 μM) increased the V_{max} 15-fold and also increased the affinity (decreased the K_m) for fructose-6-P 167-fold. From the Michaelis-Menten equation these two effects can be seen to combine to give more than a thousand-fold increase in reaction velocity at 0.12 mM fructose-6-P, which is the K_m of the activated form of the enzyme at optimum pH. In contrast, fructose-2,6-P_2 increases the affinity of mammalian ATP-dependent phosphofructokinase for fructose-6-P, but does not affect the V_{max} (31, 35, 40).

From the above considerations, it is conceivable that even if the two enzymes (ATP-dependent and PP_i-dependent) were present in the same intracellular location, the attendant conditions would determine which of the two enzymes should have the active role in the conversion of fructose-6-P to fructose-1,6-P_2. The role of the PP_i:F6P 1-phosphotransferase could be particularly important during the early stages of germination; during these stages, a process which conserves ATP may be advantageous, since the system is still at the photosynthetic-independent stage.

Two forms of PP_i:F6P 1-phosphotransferase are present in mung beans - the large and small forms. They differ with respect to their molecular weight (large form, M_r = 340,000 and small form M_r = 170,000) and are much larger than the enzymes from either P. shermanii (M_r = 95,000; ref. 18) or E. histolytica (M_r = 83,000; ref. 23). They also differ in their stability at 55°C (large form has $t_{\frac{1}{2}}$ = 14 min whereas small form has $t_{\frac{1}{2}}$ = 26 min); K_A for fructose-2,6-P_2 (the large form has K_A = 25 nM at pH 7.8 and pH 7.0 whereas the small form has K_A = 50 nM at pH 7.8 and K_A = 140 at pH 7.0); and K_m for fructose-6-P (the large form has K_m = 0.15 mM whereas the small form has K_m = 0.50 mM at pH 7.0). Some kinetic properties are the same: K_m for PP_i, K_m for fructose-6-P at pH 7.8, K_m for fructose-1,6-P_2, K_m for P_i, and the inhibition by P_i both in the presence and absence of fructose-2,6-P_2.

A comparison of the kinetic properties of the two forms of the enzyme suggests that the large form may be the more physiologically significant form. In vitro, a presumptive evidence for the possible conversion of the large form to the small form by the action of DTT was demonstrated. DTT was also found to be deleterious to the stability of the enzyme. Light and DTT treatment had been shown to inactivate glucose-6-P dehydrogenase (152) and ATP-dependent phosphofructokinase (153) in peas, and to activate a number of enzymes of photosynthetic metabolism (154-159). It is therefore probable that the action of DTT on PP_i:F6P 1-phosphotransferase simulates a possible in vivo reductive process mediated by light. A possible inactivation of PP_i:F6P 1-phosphotransferase by light would be consistent with

preventing the breakdown of hexose-phosphates and the storage of carbohydrates during photosynthesis.

The detection of the two forms of PP_i:F6P 1-phosphotransferase, which are both catalytically active, has never been shown for the enzyme from other sources. It merits future studies by purifying the two forms to homogeneity and further characterizing their properties. In addition, the formation of fructose-2,6-P_2 and the mechanism of control of its levels should also be studied. In animal tissue the levels of fructose-2,6-P_2 is under hormonal control (66a, 66b). It is tempting to speculate that a hormonal mechanism of control may also occur in plants.

A survey of the occurrence of PP_i:F6P 1-phosphotransferase in plants indicates that the enzyme is widespread among plant species. The enzyme could be found in various plant parts: seed, leaves, bulb roots, stalks and fruits. Likewise, the activator fructose-2,6-P_2 is also widespread among plant species. It is present in seeds, leaves, bulb, roots and fruits. The widespread occurrence of both the PP_i:F6P 1-phosphotransferase and the activator fructose-2,6-P_2 supports the idea (138, 139) that PP_i:F6P 1-phosphotransferase is instrumental in energy metabolism in plants.

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