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Characterization of Adenosine Diphosphate Glucose Pyrophosphorylases from Developing Maize Seeds[1,2,3]

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ABSTRACT

Electrophoretic examination of 22-day-old, normal maize (*Zea mays* L.) endosperm extracts revealed two zones of adenosine diphosphate glucose pyrophosphorylase activity. The enzymes are identical in terms of K*m* for glucose 1-phosphate and the effect of 3-phosphoglyceric acid on apparent K*m* for glucose 1-phosphate. Both enzymatic activities increase with increasing doses of the functional alleles at the *shrunken-2* and *brittle-2* loci. Molecular weight differences between the two electrophoretic species were inferred from sucrose gradient centrifugation. It is suggested that the two bands of activity represent different aggregation states of the same enzyme because under different extraction conditions, only one enzyme is found. Molecular weight estimates of 237,000 and 253,000 were obtained for the smaller enzyme. It is suggested that this enzyme is an aggregate of several subunits. Comparison of the embryo and endosperm pyrophosphorylases showed the embryo activity to be more heat stable and probably independent of direct *shrunken-2* or *brittle-2* control.

ADP-glucose pyrophosphorylase (ADP:α-D-glucose-1-phosphate adenyltransferase) has recently become of interest in investigations concerned with starch synthesis in the developing maize endosperm. Tsai and Nelson (11) have shown that the enzymatic lesion associated with two starch-deficient mutants, *sh2*[5] and *bt2,* involves ADP-glucose pyrophosphorylase. Also, Dickinson and Preiss (3) have suggested that this enzyme may have a regulatory role in starch synthesis in maize endosperm.

Several observations have suggested that there may be multiple forms of this enzyme in the endosperm. First, enzymatic assay of endosperm ADP-glucose pyrophosphorylase revealed that both the *sh2* and *bt2* mutants examined contained low activity (4) and were not devoid of activity as originally reported. In this laboratory, 14 *sh2* and *bt2* mutants of independent origin have been examined and all were found to contain low pyrophosphorylase activity. A possible explanation is that there is a second pyrophosphorylase, making up 5 to 10% of the total activity, which is independent of allelic state at the *sh2* and *bt2* loci. Weaver *et al.* (13) reported two electrophoretically separable forms of maize endosperm ADP-glucase pyrophosphorylase although both were reported to be under the control of the *sh2* and *bt2* loci.

These observations prompted an investigation of the number of different ADP-glucose pyrophosphorylases. Electrophoretic separations reveal two zones of ADP-glucose pyrophosphorylase activity, but upon closer examination, the multiple forms seem to represent different aggregation states of a common protein. The possibility that the presumed aggregation affects the catalytic and allosteric properties was examined, and negative results were obtained. Thus, the physiological significance of the presumed aggregation is unknown. On the basis of evidence at hand, the multiple banding pattern can be considered artifactual.

MATERIALS AND METHODS

Substrates and Reagents. The trisodium salt of EDTA, DTT, GSH, 3-PGA, HEPES, ATP, glucose-1-P, β-galactosidase grade IV, MTT tetrazolium, and phenazine methosulfate were purchased from Sigma. Special enzyme grade ammonium sulfate was obtained from Schwarz/Mann and BSA from Nutritional Biochemicals Corporation. Carbon-14 glucose-1-P, PPO, and dimethyl POPOP were obtained from New England Nuclear. Protamine sulfate and pyruvate kinase were purchased from Calbiochem. The DEAE-celluloses (DE-52 and DE-81) were Whatman products. Alkaline phosphatase (BAPSF) was obtained from Worthington.

Biological Materials. Maize (*Zea mays* L.) kernels were harvested from field-grown plants at 22 days postpollination, the time at which the rate of starch synthesis and activities of most starch synthetic enzymes are greatest (12). Freezing and storage did not affect ADP-glucose pyrophosphorylase activity. All material examined resulted from controlled pollinations.

Normal (nonmutant) kernels were the F_2 progeny from the hybrid W64A × 182E. The mutants *sh2-R* (reference) and *bt2-R* (reference) are the mutant alleles which were first described at the *sh2* and *bt2* loci, respectively. Other alleles reported herein were shown to be *sh2* or *bt2* alleles in this laboratory. Most *sh2* and *bt2* stocks used in these studies were

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[5] Abbreviations: *sh2: shrunken-2; bt2: brittle-2;* DTT: dithiothreitol; MTT tetrazolium: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; 3-PGA: 3-P-glyceric acid.

backcrossed three to four times into the inbred W22 and subsequently recovered in a homozygous condition.

In this report, the allelic designation will denote the genotype of homozygous material. For example, endosperms of the genotype *sh2-R/sh2-R/sh2-R* will be referred to simply as *sh2-R* endosperms. Utilizing the fact that the triploid endosperm receives two genomes from the female and one from the male, the 16 genotypes were generated from normal, the single mutants *sh2-R* and *bt2-D*, and the double mutant, *sh2-R;bt2-R*.

Preparation of Homogenates. The extraction and dialysis buffers used were those reported by Dickinson and Preiss (3). The extraction buffer was 0.03 M potassium phosphate, pH 7, 0.5 mM EDTA (trisodium salt), 5 mM $MgCl_2$, and 0.1 mM DTT. The dialysis buffer was 10 mM potassium phosphate, pH 7, containing 0.1 mM GSH.

Kernels were blended in equal volume of cold, freshly prepared extraction buffer using a VirTis 45 homogenizer. The homogenate was passed through two layers of cheesecloth and centrifuged 20 min at 39,100*g* in an SS-34 rotor. Subsequent steps were done at 0 to 5 C.

When electrophoretic mobility was examined, solid ammonium sulfate (37.2 g/100 ml) was added slowly to the stirred crude extract. The mixture was equilibrated 45 min, then centrifuged 15 min at 20,200*g*. The resulting pellet was dissolved in one-tenth starting volume of dialysis buffer and dialyzed 15 hr. The dialyzate was centrifuged 15 min at 20,200*g* and the supernatant fluid was used as the enzyme source.

For preparation of embryo ADP-glucose pyrophosphorylase, embryos were dissected from the kernels and blended in 3 volumes of extraction buffer in a 7-ml ground glass homogenizer. The mixture was centrifuged 20 min at 39,100*g* in an SS-34 rotor. The supernatant fluid was used as the enzyme source. Assays were done for 30 min in the presence of 20 mM 3-PGA and 1.5 mM potassium phosphate.

Separation of Endosperm ADP-glucose Pyrophosphorylases. To separate the two enzymes, 0.3 volume of 1% (w/v) protamine sulfate was added slowly to the stirred crude extract. The suspension was kept in ice 15 min, then was centrifuged 15 min at 20,200*g*. The resulting supernatant fluid contained the slower migrating enzyme (A). The pellet was dissolved in one-half the initial volume of 0.3 M potassium phosphate buffer (pH 7) by stirring with a glass rod. The suspension was stored in ice for 20 min, then centrifuged 15 min at 20,200*g*. Solid ammonium sulfate (21 g/100 ml) was added to the supernatant fluid as described above. The pellet was dissolved in extraction buffer to obtain a crude preparation of enzyme B.

To determine the effect of gene dosage on enzymatic activity, the two endosperm enzymes were prepared as described above. Assays were done in triplicate using nondialyzed preparations. In each experiment, normal enzyme was extracted and used for comparison.

Measurement of Enzymatic Activities. ADP-glucose synthesis was measured, with modification, using the methods of Dickinson and Preiss (3). The reaction mixture contained 2 μmoles of HEPES, pH 8, 2 μmoles of $MgCl_2$, 0.2 μmole of ATP, 0.2 μmole of glucose-1-P (3–4 × 10^5 cpm/μmole), 50 μg of BSA, 2 μmoles of 3-PGA, and enzyme in a final volume of 0.1 ml. In control tubes, water was substituted for ATP. The reaction was run at 37 C, usually for 10 min, and was terminated by boiling for 1 min. To the cooled reaction tubes, 0.03 mg of alkaline phosphatase was added and the tubes were incubated 1 to 2 hr at 37 C.

The separation of ADP-glucose and glucose followed the method of Hopper and Dickinson (5). Fifteen 16-mm circles were drawn on a 145-mm circle of DEAE-cellulose paper. A 20-μl reaction aliquot was placed on each 16-mm circle. The paper was irrigated using a 3-liter Büchner-type funnel fitted with a coarse-fritted disc. The paper was placed under a plastic cover which exposed the 15 samples to the water flow. Four liters of twice-distilled H_2O were passed through at 16 l/hr. The proportion of ADP-glucose retained is dependent on the amount of reaction mixture added but independent of the amount of product produced. Under these conditions, 85% ADP-glucose retention is found. The paper was dried and the individual samples were placed in scintillation vials. The scintillation fluid contained 50 mg of dimethyl POPOP and 6 g of PPO/l toluene. The vials and fluid were reused.

One enzyme unit is defined as 1 μmole of ADP-glucose formed in 10 min under the above conditions.

Pyruvate kinase was assayed using a modified version of Leloir and Goldemberg (6). The β-galactosidase assay was that of Zipser (14). For alkaline phosphatase, the reaction mixture contained 1 ml of 10 mM tris-acetate (pH 8) containing 1 mM *p*-nitrophenyl phosphate and 15 μl of enzyme. After 30 min at 37 C, the reaction was stopped by addition of 1 ml of 1 M Na_2CO_3. Absorbance at 420 nm was determined.

All protein determinations were done by the method of Lowry *et al.* (7). BSA was used as a standard.

Electrophoresis. A disc electrophoresis system using 7.5% polyacrylamide running gels (2) was used for separation. The cathodic buffer was 2.5 mM tris and 5 mM glycine, pH 8.9, and the anodic buffer was 2.5 mM tris and 20 mM glycine, pH 8.6.

The method of Salamini *et al.* (10) for glucose phosphate isomerase detection was modified to reveal pyrophosphorolysis of ADP-glucose. A 50-ml reaction mixture contained 2.5 μmoles of HEPES, pH 8, 240 μmoles of $MgSO_4$, 60 μmoles of tetrasodium pyrophosphate, 65 μmoles of 3-PGA, 24 μmoles of ADP-glucose, 6 mg of NADP, 1 mg of MTT tetrazolium, 0.6 mg of phenazine methosulfate, 10 units of glucose-6-P dehydrogenase, and 10 units of phosphoglucomutase. Incubation was done in the dark.

ADP-glucose synthesis was detected with the staining procedure outlined by Weaver *et al.* (13). In both assays, gels were incubated with 2 ml of reaction mixture at 37 C until zones of activity appeared.

The activity detected is inferred to be ADP-glucose pyrophosphorylase because (*a*) both methods detect the same bands, and (*b*) the bands in the former method are dependent on ADP-glucose and pyrophosphate and the bands in the latter procedure are dependent upon ATP and glucose-1-phosphate.

Molecular Weight Determination. The faster-migrating enzyme (B) was prepared as described under separation of forms. *E. coli* β-galactosidase and pyrophosphorylase were dialyzed separately against the dialysis buffer. Linear 5 to 20% (w/v) sucrose gradients (4.6 ml) were prepared in dialysis buffer. Centrifugation was done in a Spinco Model L centrifuge with an SW39 rotor. Procedures for gradient preparation, removal of samples, and mol wt calculation were those of Martin and Ames (8).

A 56-ml Sephadex G-200 column, equilibrated in 0.1 M potassium phosphate (pH 7) containing 0.1 mM GSH, separated a mixture of 20 units of β-galactosidase (*E. coli*, mol wt = 520,000), 15 units of pyruvate kinase (rabbit muscle mol wt = 237,000), 4 units of alkaline phosphatase (*E. coli*, mol wt = 80,000), and 50 units of the pyrophosphorylase B. One-milliliter fractions were collected at 5 ml/hr.

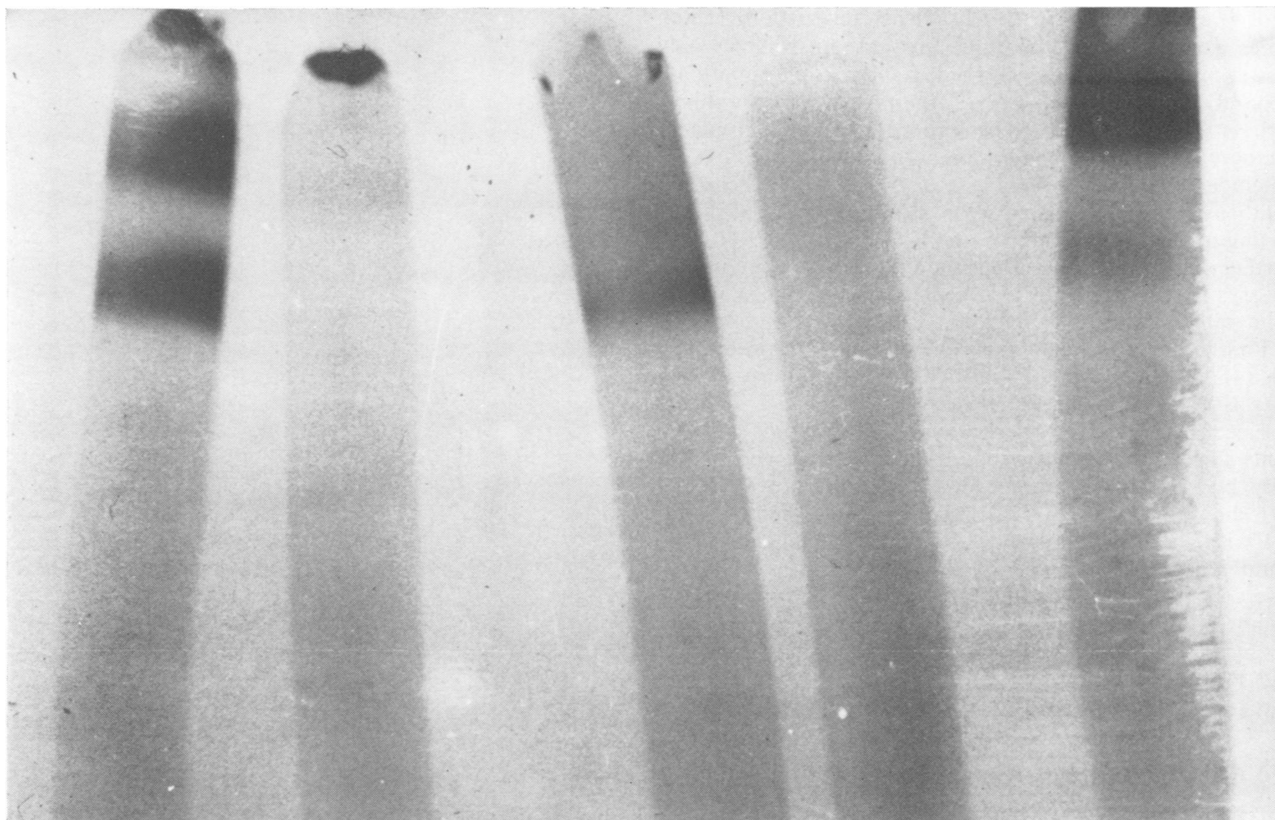

FIG. 1. Detection and separation of maize ADP-glucose pyrophosphorylase activities. Activity was detected by observation of glucose-1-P synthesis. Migration is from top to bottom. From left to right the gels are: (*a*) crude homogenate stained for activity; (*b*) control (minus ADP-glucose) for crude homogenate; (*c*) activity bound to protamine sulfate; (*d*) control for above; and (*e*) activity not bound to protamine sulfate (control not shown).

RESULTS AND DISCUSSION

Detection and Characterization of Maize Endosperm ADP-glucose Pyrophosphorylase. Electrophoresis and subsequent enzymatic staining of normal, nonmutant enzyme preparations reveal two bands of ADP-glucose pyrophosphorylase activity (Fig. 1A). Enzyme A migrates at roughly one-half the rate of enzyme B. It was found (Fig. 1) that only the faster migrating enzyme would bind to protamine sulfate when the crude homogenate is treated with protamine sulfate as described by Dickinson and Preiss (3). These workers discarded the resulting pellet and thus may have examined only pyrophosphorylase A. Differential binding to protamine sulfate under these conditions was not expected because Dickinson and Preiss (3) show no loss in total activity resulting from this purification step. The following data are in agreement with their findings. On a gram basis, 2.45 units of activity were found in the crude extract and 2.94 units after protamine sulfate treatment. However, activity bound to protamine sulfate was 2.69 units/g of tissue. Comparison of the activity in the supernatant fluid before and after protamine-sulfate treatment shows no apparent loss in activity, although the amount of activity bound is approximately equal to the amount remaining in the supernatant. Moreover, these data show that enzymatic assay of crude preparations of ADP-glucose pyrophosphorylase measures only about one-half the total activity. Whether protamine sulfate removed an inhibitor of pyrophosphorylase is unknown.

Activity profiles resulting from sucrose gradient centrifugation showed that the electrophoretic mobility difference is attributable, at least in part, to mol wt differences. Figure 2 shows enzymatic profiles obtained from the crude homogenate (pyrophosphorylases A and B) and a preparation containing only pyrophosphorylase B. Two preparations which contained only pyrophosphorylase A yielded no activity after centrifugation. The reason for the loss of pyrophosphorylase A activity in this type of preparation is presently unknown. Electropho-

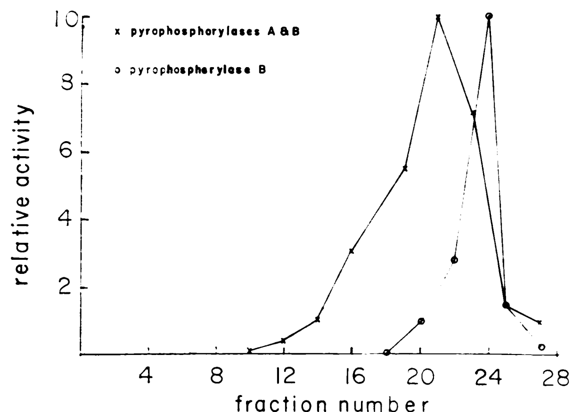

FIG. 2. Enzyme profiles of crude homogenate and pyrophosphorylase B from centrifugation through a linear 5 to 20% sucrose gradient. Centrifugation was done in a Spinco Model L with an SW39 rotor at 21,890 rpm for 940 min. For the crude homogenate, the peak tube contained 2.2 units of enzyme/ml, and the peak tube of form B contained 1.3 units/ml.

resis of fraction 21 in the two gradients revealed that both enzymes were present in the crude preparation, whereas only the faster-migrating form was found in the pyrophosphorylase B preparation. As seen in Figure 2, the presence of pyrophosphorylase A in a preparation containing enzyme B displaces the activity peak toward the bottom of the centrifuge tube. It is concluded that pyrophosphorylases A and B differ in mol wt, with pyrophosphorylase A, the electrophoretically-slower migrating form, being larger.

Further characterization suggests that the two pyrophosphorylases observed do not represent separate molecular species but rather different aggregation states of a common protein. First of all, Tsai and Nelson (11) and Dickinson and Preiss (4) have shown that ADP-glucose pyrophosphorylase activity is dependent on the allelic state of the *bt2* and *sh2* loci. Electrophoretic characterization of several *sh2* and *bt2* mutants showed that both pyrophosphorylases are reduced in activity by mutation at either locus. Furthermore, Tables I and II show that the activities of both enzymes generally increase with increasing doses of the functional alleles at the *sh2* and *bt2* loci. Exceptions to this pattern arise when the genetic constitution of either locus is varied in the presence of 3 mutant alleles at the other locus. The control of both enzymes by *sh2* and *bt2* could be explained if pyrophosphorylases A and B were separate entities and if the *sh2* and *bt2* loci were regulatory for, or had an indirect effect on, both enzymes. However, characterization of pyrophosphorylase B from several *sh2* and *bt2* mutants (Hannah and Nelson, in preparation) leads to the conclusion that these loci are structural genes for the enzymes. Because of stability difficulties, similar analyses of pyrophosphorylase A from these mutants have not been successful. Since pyrophosphorylase A activity is controlled by the *sh2* and *bt2* loci and shows *sh2* and *bt2* dosage dependence, it seems reasonable that these loci are structural genes for this enzyme as well.

Secondly, pyrophosphorylases A and B seem to be interconvertible. This is inferred from experiments designed to reconcile the differences in electrophoretic banding patterns observed by Weaver *et al.* (13) and by us (Fig. 1). Weaver *et al.* (13) observed two very closely migrating enzymes which correspond to enzyme B reported here. Pyrophosphorylase A was not observed. We also have observed two zones of activity at the B position and have noted that this occurs when extraction and dialysis buffers age for more than 2 days. Figure 3 shows the banding pattern obtained when the enzyme was prepared and electrophoresed according to Weaver *et al.* (13). The extraction buffer was prepared the day of extraction and electrophoresis. In contrast to their findings, only one enzyme is observed. Electrophoresis (not shown) of activity extracted by the method of Weaver *et al.* (13) and by the method presented here and electrophoresis of a mixture of the two enzyme preparations show that this band corresponds to band B reported

Table I. *Relative Pyrophosphorylase A Activity as a Function of Sh2 and Bt2 Dosage*

One hundred per cent equals 1.56 units/gm kernel.

Bt2 Alleles	*Sh2* Alleles			
	3	2	1	0
3	100	87	58	22
2	49	84	56	18
1	49	40	46	16
0	15	13	13	16

Table II. *Relative Pyrophosphorylase B Activity as a Function of Sh2 and Bt2 Dosage*

One hundred per cent equals 2.17 units/gm kernel.

Bt2 Alleles	*Sh2* Alleles			
	3	2	1	0
3	100	60	42	4
2	61	38	24	6
1	23	29	40	4
0	5	2	8	5

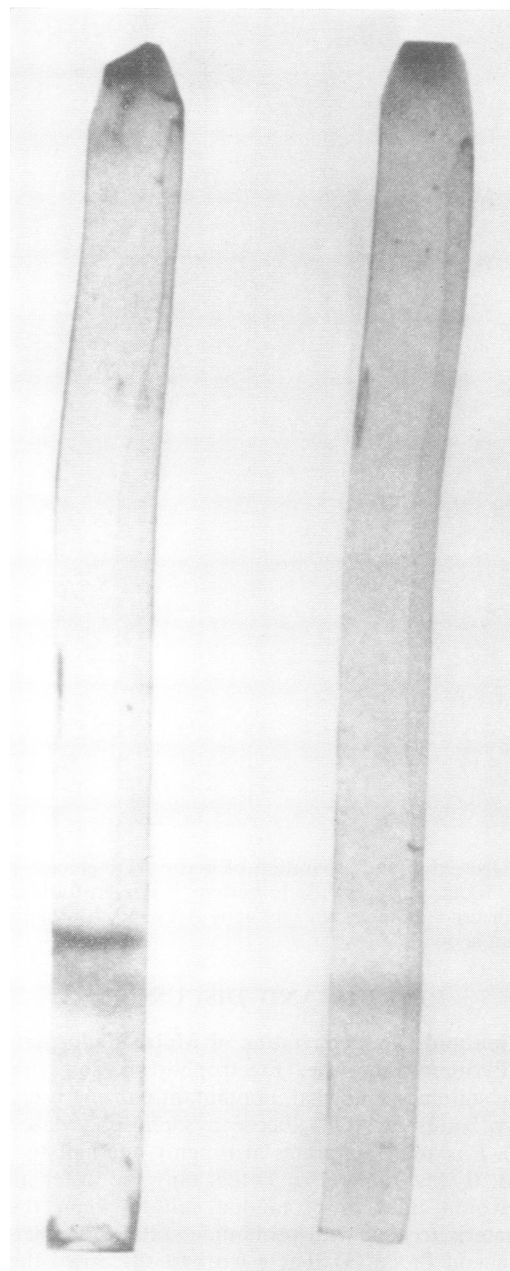

FIG. 3. Banding pattern of ADP-glucose pyrophosphorylase activity obtained when extraction and electrophoresis are done as described by Weaver *et al.* (13). Migration is from top to bottom. Activity was detected by observation of ADP-glucose synthesis. Left gel was stained in the presence of complete reaction mixture, whereas reaction mixture for right gel lacked glucose-1-P.

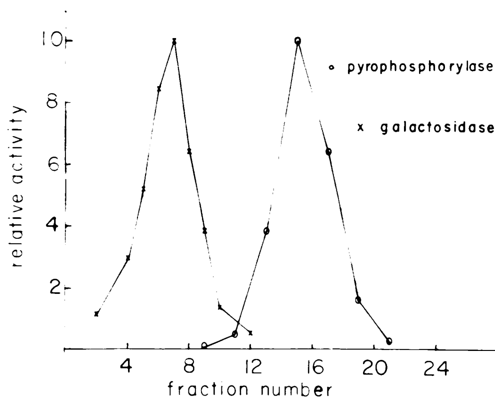

FIG. 4. Enzymatic profiles resulting from linear (5–20%) sucrose gradient centrifugation of a mixture of *E. coli* β-galactosidase and ADP-glucose pyrophosphorylase B. Protein migration is from right to left. Centrifugation was done in a Spinco Model L with an SW39 rotor for 1140 min at 27,112 rpm. The peak tube of pyrophosphorylase contained 3.4 units/ml.

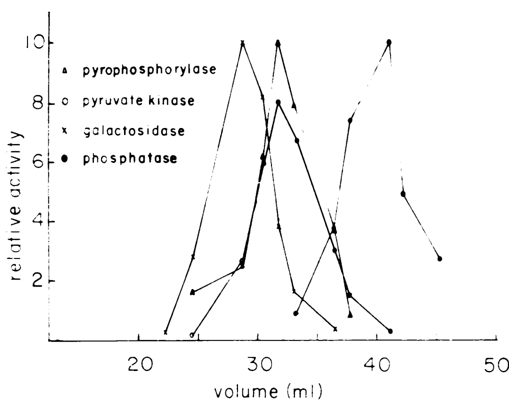

FIG. 5. Gel filtration (Sephadex G-200) profiles obtained from a mixture of β-galactosidase, pyruvate kinase, alkaline phosphatase, and pyrophosphorylase B. Bed volume equals 56 ml.

above. Thus, subtle differences in composition affect the banding pattern at position B.

In agreement with the observations of Weaver *et al.* (13), pyrophosphorylase A was not observed (Fig. 3). The loss of pyrophosphorylase A could be attributable to preferential denaturation or inhibition by the tris-HCl buffer used by Weaver *et al.* (13). Alternatively, the two pyrophosphorylases may be interconvertible. Preliminary experiments have shown that 83% of the activity extracted in the phosphate buffer was recovered in tris-HCl, suggesting that conversion of all enzyme into the electrophoretic species has occurred.

The possibility that the presumed aggregation affects the properties of pyrophosphorylase was partially examined. Dickinson and Preiss (3) demonstrated that 3-PGA lowers the apparent K*m* for glucose-1-P from 0.1 mM to 0.05 mM. It should be recalled that Dickinson and Preiss (3) may have examined only the form we have designated as pyrophosphorylase A. In view of this, the K*m* values of both enzymes were determined in the presence and absence of 3-PGA. For enzyme A, K*m* estimates of 0.1 mM (−3-PGA) and 0.05 mM (+3-PGA) were obtained. Similarly, estimates of 0.09 mM (−3-PGA) and 0.04 mM (+3-PGA) were obtained for enzyme B. Thus, both enzymes interact similarly with 3-PGA. Therefore aggregation, if it occurs, does not appreciably affect this enzymatic property.

The effect of 3-PGA on pyrophosphorylase of developing endosperm was studied because Dickinson and Preiss (3) have suggested that this activation is physiologically important. Alternatively, if different ADP-glucose pyrophosphorylases exist in different plant tissues and if the different structural genes for these enzymes arose by gene duplication, the observed 3-PGA activation in the developing endosperm may not be physiologically significant but rather may represent a property of the enzyme that stems from its evolutionary history.

The mol wt of the faster-migrating pyrophosphorylase was estimated. Figure 4 presents the sucrose gradient profiles obtained from a mixture of *E. coli* β-galactosidase and the pyrophosphorylase B. A mol wt of 253,000 was calculated by the procedure of Martin and Ames (8). The mol wt was also estimated by gel filtration. As shown in Figure 5, the pyrophosphorylase activity profile was superimposed on the pyruvate kinase profile. Thus, the mol wt is estimated to be 237,000.

In summary, there seems to be only one major ADP-glucose pyrophosphorylase in maize endosperm, and this enzyme seems to be capable of aggregation which yields the multiple banding pattern seen upon electrophoresis following extraction in a phosphate buffer. The different multiple banding pattern reported by Weaver *et al.* (13) is also artifactual and dependent on conditions of extraction. The relatively large mol wt of pyrophosphorylase B suggests that this enzyme is itself an aggregation of several subunits. If the *sh2* and *bt2* loci are both structural genes for this enzyme (Hannah and Nelson, in preparation), this enzyme must then be at least a dimer. A structural role for these genes suggests that activity level in a *sh2*;*bt2* double mutant should be much lower than that found in either single mutant. Analysis of one double mutant, *sh2-R*; *bt2-R*, showed that the activity levels are equal to those found in *sh2-R* (Tables I and II). This could be explained by protein interactions which are akin to those involved in intragenic complementation or to the presence of a second pyrophosphorylase which is independent of *sh2* and *bt2* function yet electrophoretically indistinguishable from the major enzyme.

Embryo ADP-glucose Pyrophosphorylase. The question of whether the maize endosperm and embryo contain the same ADP-glucose pyrophosphorylase was raised initially by Dickinson and Preiss (4). Preiss *et al.* (9) concluded, on the basis of different heat stabilities, that the two tissues contain different enzymes although their observations could also be explained by different levels of protective substances in the two extracts. Our results (not shown) confirm their observation of greater apparent thermostability for the enzyme extracted from the embryo, and we agree that in all probability the embryo enzyme is a different enzyme than the endosperm form. This conclusion is based not only on the thermostability studies but the finding that the *bt2* and *sh2* mutations which drastically affect the endosperm do not substantially affect the amount of embryo enzyme activity (Table III). In this regard, our results do not agree with those of Dickinson and Preiss (4) who found a substantial reduction, compared to normal, in *bt2* embryo activity (expressed on a protein basis) and an in-

Table III. *The Effects of Mutation of the sh2 and bt2 Loci on Embryo Fresh Weight, Water-soluble Protein, and ADP-glucose Pyrophosphorylase Activity*

Data are expressed on an embryo basis.

Genotype	Weight	Protein	Enzyme Units × 10^3
	mg		
Normal	8.01	0.163	6.08
sh2-D	6.40	0.154	3.36
bt2-D	7.12	0.175	5.52

crease in *sh2* embryo activity on a weight basis. The discrepancy in the two sets of data may be attributable to differences in genetic background, but this possibility can be determined only when isogenic stocks become available. On the basis of these preliminary data, it seems apparent that if *sh2* affects embryo activity, the effect of *sh2* substitution on embryo activity is not as severe as the effect on endosperm activity (Tables I and II), and we think that the embryo enzyme is independent of direct control by the *bt2* and *sh2* loci.

The tissue specificity of ADP-glucose pyrophosphorylases agrees with an emerging pattern for maize starch enzymes. Akatsuka and Nelson (1) demonstrated that the endosperm starch-bound glucosyl transferase is under the control of the *waxy* locus whereas the embryo counterpart is not. Comparison of various kinetic parameters suggests that the two enzymatic activities are different. In the case of glucose phosphate isomerase activity, three isoenzymes have been reported, only one of which is found in both tissues (10). One can envisage the advantages of this tissue specificity if the cytoplasmic signals which regulate starch content are different in the two tissues.

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