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# A Unique Adenosine Diphosphoglucose Pyrophosphorylase Associated with Maize Embryo Tissue[1]

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## ABSTRACT

**A comparison of heat stabilities and various kinetic properties between the adenosine diphosphoglucose pyrophosphorylases isolated from endosperm and embryo tissues from starchy maize seeds indicates that the adenosine diphosphoglucose pyrophosphorylase associated with the embryo is distinct from the enzyme isolated from the endosperm. The embryo enzyme is more stable to incubation for 5 minutes at 60 C while the endosperm enzyme is labile to this treatment. Both enzymes are activated by glycerate-3-P. The embryo enzyme is more sensitive to inhibition by phosphate than is the endosperm enzyme. Glycerate-3-P, which reverses the inhibition of the endosperm enzyme by phosphate, has little effect on the phosphate inhibition of the embryo enzyme. Other kinetic studies distinguish the two enzymes.**

A recent report (5) indicated the presence of ADP-glucose pyrophosphorylase in the embryo and endosperm tissues of maize seeds harvested in the milky stage, 22 days after self-pollination. The ADP-glucose pyrophosphorylase activity present in the embryo tissue of starchy maize (DeKalb 805) was only 1% of the total activity in the seed. However, the activity present in the embryo tissue of shrunken-2 mutant maize seeds was about 16% of the total ADP-glucose pyrophosphorylase activity in the seed since there was a drastic decrease in enzyme activity (about 92%) in the endosperm when compared to starch maize endosperm. As activities in the mutant and normal embryo tissues were about the same it was postulated that the mutation in $Sh_2$[2] affected only the enzyme activity present in the endosperm but not in the embryo (5). This report will show that the kinetic and heat sensitivity properties of the ADP-glucose pyrophosphorylase present in the embryo of starchy maize are quite different than those of the endosperm ADP-glucose pyrophosphorylase. The properties of the embryo ADP-glucose pyrophosphorylase from $Sh_2$ seeds are found to be very similar to the properties of the starchy maize embryo enzyme.

## MATERIALS AND METHODS

Seeds of starchy maize (variety DeKalb 805) and $Sh_2$ were harvested in the milky stage, 22 days after self-pollination, and stored at −10 C until use. Methods of assaying ADP-glucose pyrophosphorylase and the reagents used are similar to those reported earlier (4). Protein was determined according to Lowry *et al.* (7).

The following solvent systems were used for descending paper chromatography on Whatman No. 1 filter paper: (*a*) solvent A, 95% ethanol–M ammonium acetate, pH 3.8 (5:2); (*b*) solvent B, 95% ethanol–M ammonium acetate, pH 7.5 (5:2); (*c*) solvent C, isobutyric acid–1 M $NH_4OH$–0.1 M EDTA, pH 7.2, (10:6:0.16).

**Assay of ADP-Glucose Pyrophosphorylase. Assay A.** Pyrophosphorolysis of ADP-glucose was determined by the formation of ATP-$^{32}P$ from $PP_i$ (9). The reaction mixture contained 20 μmoles of Hepes (*N*-2-hydroxy-ethylpiperazine-*N'*-2 ethanesulfonic acid) buffer, pH 8.0, 50 μg of crystalline bovine serum albumin, 0.2 μmole of ADP-glucose, 0.5 μmole of $^{32}P$-$P_i$ ($1–4 \times 10^6$ cpm/μmole), 2 μmoles of $MgCl_2$, 2.5 μmoles of NaF and enzyme in a final volume of 0.25 ml. Glycerate-3-P (2.0 μmoles) was added to determine enzyme activity in the presence of activator. The mixture was incubated 10 min at 37 C, and the reaction was terminated by addition of 3 ml of cold 5% trichloroacetic acid. The ATP-$^{32}P$ formed was adsorbed onto Norit A and counted in a gas flow counter.

**Assay B.** Glucose-$^{14}C$-1-P was used to measure synthesis of ADP-glucose-$^{14}C$ (6). The reaction mixture contained 15 μmoles of Hepes buffer, pH 8.0, 50 μg of bovine serum albumin, 2 μmoles of $MgCl_2$, 0.2 μmole of ATP, 0.1 μmole of glucose-$^{14}C$-1-P ($6.87 \times 10^5$ cpm/μmole) and enzyme in a final volume of 0.2 ml. 3-PGA (3 μmoles) was added when enzyme was assayed in the presence of activator. The mixture was incubated for 30 min at 37 C and the reaction was terminated by heating for 40 sec in a boiling water bath and assayed as previously described. All kinetic measurements were done under conditions where the enzyme activity was proportional to both time of incubation and enzyme concentration.

**Partial Purification of the Embryo ADP-Glucose Pyrophosphorylase.** Frozen seeds of DeKalb 805 maize that were harvested in the milky stage, 22 days after self-pollination, were dissected and the embryo (+ scutellum) were separated from the pericarp and endosperm tissues. The embryos (10 g) were frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. The powder was allowed to warm and an equal volume of buffer, pH 7.0, containing 30 mM potassium phosphate, 0.5 mM EDTA, 5 mM $MgCl_2$, and 0.1 mM dithiothreitol was added. The resulting suspension was mixed at 4 C for 20 min and then centrifuged for 20 min at 30,000*g*. The supernatant fluid was then heated for 5 min in a 65 C water bath. The denatured protein was removed by centrifugation and the heat-treated supernatant fluid was subjected to ammonium sulfate fractionation with the solid salt. The fraction precipitating between 0 to 0.45 saturation contained about 90% of the ADP-glucose pyrophosphorylase activity. The precipitate was dissolved in 0.5 ml of 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM GSH. Table I summarizes the purification procedure that gives about a 16-fold enrichment of the enzyme

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[2] Abbreviations: 3-PGA: glycerate-3-P; $Sh_2$: shrunken-2.

activity with respect to protein concentration and a 78% recovery of the total activity. One unit of enzyme activity is that amount of enzyme that forms 1 μmole of ATP in 10 min under the conditions of assay A.

The ADP-glucose pyrophosphorylase associated with the embryo tissue from $Sh_2$ maize seeds was partially purified in the same manner by the above procedure and similar results in purification were achieved.

## RESULTS

**Heat Stability of the Embyro ADP-Glucose Pyrophosphorylase.** Figure 1 compares the heat stability of the ADP-glucose pyrophosphorylase present in the embryo extract with the ADP-glucose pyrophosphorylase present in endosperm extract. Whereas a 5 min heat treatment at 65 C of the endosperm enzyme resulted in greater than 90% loss of the activity, the same treatment gave only about a 30% decrease of the embryo enzyme activity. In other experiments, the loss of ADP-glucose pyrophosphorylase activity in the embryo extract by heat treatment at 65 C resulted in only 20% loss of activity. Heat treatment at 50 C resulted in only a 15% decrease in the embryo enzyme activity but in a 60% decrease in the endosperm enzyme activity. The ADP-glucose pyrophosphorylase activity present in the embryo tissue is more stable to heat than the activity present in the endosperm tissue. The 20 to 30% decrease in activity observed in

Table I. *Partial Purification of the ADP-Glucose Pyrophosphorylase from DeKalb 805 Seed Embryos*

| Fraction | Volume | Total Units | Protein | Specific Activity |
|---|---|---|---|---|
| | *ml* | | *mg/ml* | *units/mg protein*[1] |
| Crude extract | 13.6 | 21.6 | 5.2 | 0.31 |
| Heat-treated supernatant fluid | 13.0 | 16.9 | 0.73 | 1.7 |
| Ammonium sulfate | 0.55 | 16.9 | 6.3 | 4.9 |

[1] 1 unit = 1 μmole of ATP formed in 10 min under the conditions of assay A in the presence of 8 mM 3-PGA.

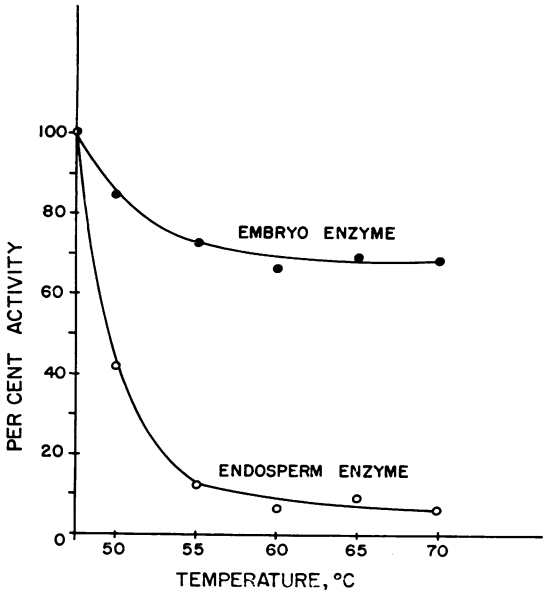


FIG. 1. Heat stabilities of the embryo and endosperm ADP-glucose pyrophosphorylases. Per cent activity pertains to the conditions of assay A (pyrophosphorolysis of ADP-glucose in presence of 8 mM 3-PGA). Aliquots of 0.5 ml of crude extract of embryo or endosperm tissue prepared by freezing in liquid $N_2$, and extraction with buffer, as indicated in the text, were heated for 5 min at the temperatures indicated in the figure. The denatured protein was separated by centrifugation and appropriate dilutions of the supernatant solutions of the heat-treated extracts were assayed for activity.

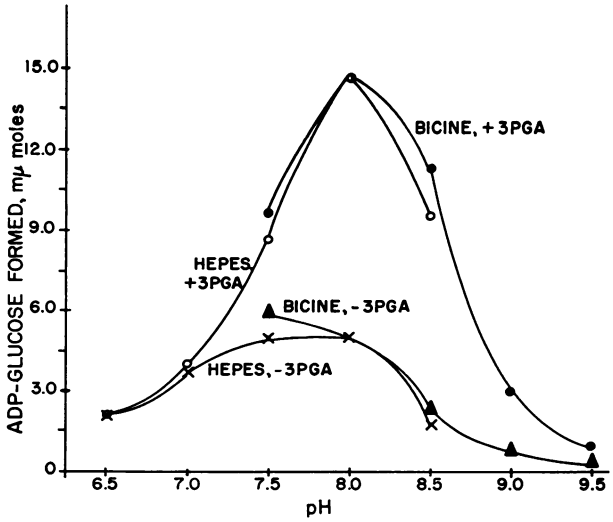


FIG. 2. pH optimum of the embryo ADP-glucose pyrophosphorylase. The conditions were those of assay B in the absence or presence of 10 mM 3-PGA.

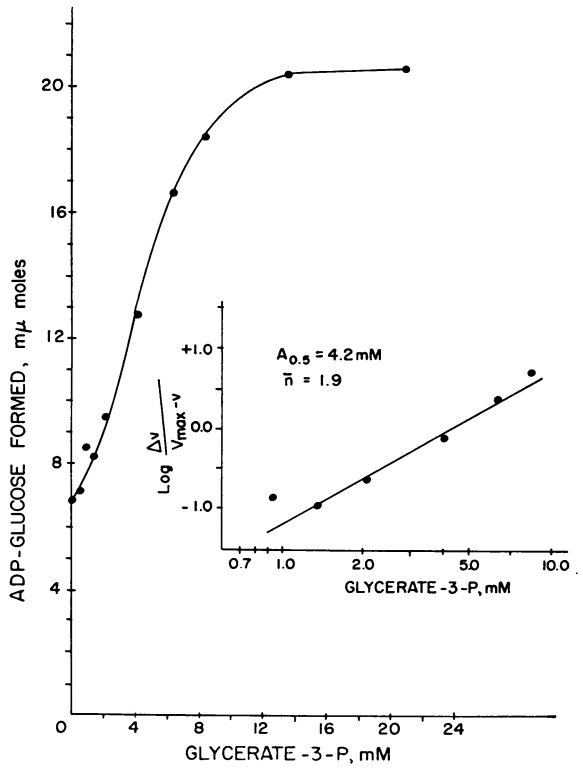


FIG. 3. The effect of 3-PGA on ADP-glucose synthesis. The conditions are those of assay B. $A_{0.5}$ represents the concentration of activator causing 50% of the maximal stimulation. The inset is a plot of the data according to the Hill equation (2, 3). The increment of velocity due to addition of 3-PGA to the reaction mixtures is $\Delta v$. $V_{max}$ is maximal velocity and is obtained from reciprocal plots of velocity (v) versus 3-PGA concentration. The interaction coefficient or the apparent order of reaction with respect to 3-PGA is $\bar{n}$.

the embryo extract by the heat treatment may be due to the presence of two forms of ADP-glucose pyrophosphorylase in the embryo; a heat-stable form and a heat-labile form which may be similar to the ADP-glucose pyrophosphorylase present in the endosperm. It could also be explained by the presence of some contaminating endosperm tissue isolated along with the embryo tissue. Further studies are required to determine which interpretation is correct.

**pH Optimum of the Embryo Enzyme.** Figure 2 shows the pH optimum curves for ADP-glucose synthesis catalyzed by the partially purified embryo ADP-glucose pyrophosphorylase. Optimal synthesis in the presence of 3-PGA occurs at pH 8.0 both in Hepes buffer and in Bicine (*N,N'*-bis(2-hydroxyethyl)-glycine buffer. In the absence of 3-PGA the pH optimum in Hepes buffer was 7.5 to 8.0 and in Bicine buffer was pH 7.5. A previous report indicated that the maize endosperm ADP-glucose pyrophosphorylase had a broad pH optimum in the presence of Hepes buffer and 3-PGA ranging from 6.5 through 8.3 (4). Of interest is that the greatest stimulation (3- to 4-fold) of the endosperm enzyme by 3-PGA occurred in the pH range of 6.3 through 6.7 and that minimal stimulation occurred at pH 7.9 (only 1.5-fold). Figure 2 shows that for the maize embryo enzyme no stimulation of ADP-glucose synthesis by 3-PGA is observed in Hepes buffer in the range of pH 6.5 through 7.0, and that maximal stimulation by 3-PGA (3- to 5-fold) occurs in the pH range of 8.0 to 8.5. Thus, although both the endosperm and embryo ADP-glucose pyrophosphorylases may have the same pH optimum for ADP-glucose synthesis, there is a difference in the two activities with respect to the pH where maximal stimulation by 3-PGA occurs.

Optimal pyrophosphorolysis of ADP-glucose by the maize embryo enzyme occurs at pH 8.0 in Hepes buffer under the conditions of assay A in the presence or absence of the activator, 3-phosphoglycerate.

**Activation by 3-PGA.** Figure 3 shows the effect of 3-PGA concentration on the rate of ADP-glucose synthesis catalyzed by the embryo ADP-glucose pyrophosphorylase. Maximal activation occurs at 12.5 mM while 50% of the maximal stimulation occurs at 4.2 mM. The activation curve is sigmoidal in shape and not hyperbolic, and a plot of the data according to the Hill equation (2,3) gives an interaction coefficient or apparent order of reaction, $\bar{n}$, of 1.9. Previous studies with the endosperm ADP-glucose pyrophosphorylase also showed it to be activated by 3-PGA (4,5). However, the activation curve for the endosperm enzyme was not sigmoidal but hyperbolic in shape, giving an $\bar{n}$ value of one.

**Specificity of Activation.** It was reported previously that fructose-6-P and ribose-5-P stimulated the endosperm ADP-glucose pyrophosphorylase although not to the same extent as 3-PGA (4). In contrast, it was found that both fructose-6-P and ribose-5-P at either 1 mM or 15 mM did not increase the rate of ADP-glucose synthesis catalyzed by the embryo enzyme. Other compounds which did not stimulate the embryo enzyme under the conditions of assay B when tested at concentrations of 1 or 15 mM were phosphoenolpyruvate, fructose-1,6-di-P, glycerate-2-P, glucose-6-P, pyruvate, glyceraldehyde-3-P, malate, oxaloacetate, acetyl CoA, 2,3-glycerate-diP, sucrose, TPN, TPNH, DPN, and DPNH.

**Effect of 3-PGA on Kinetic Parameters.** Figure 4 shows the effect of the concentration of ATP and the effect of 3-PGA on the $Mg^{2+}$ saturation curve. In absence of activator, lower concentrations of $MgCl_2$ were required for both maximal activity (5 mM) and for half-maximal activity (1.4 to 2 mM). In the presence of 12.5 mM 3-PGA about 4 to 5 mM $MgCl_2$ was required for half-maximal activity and 10 mM $MgCl_2$ gave maximal rates of syn-

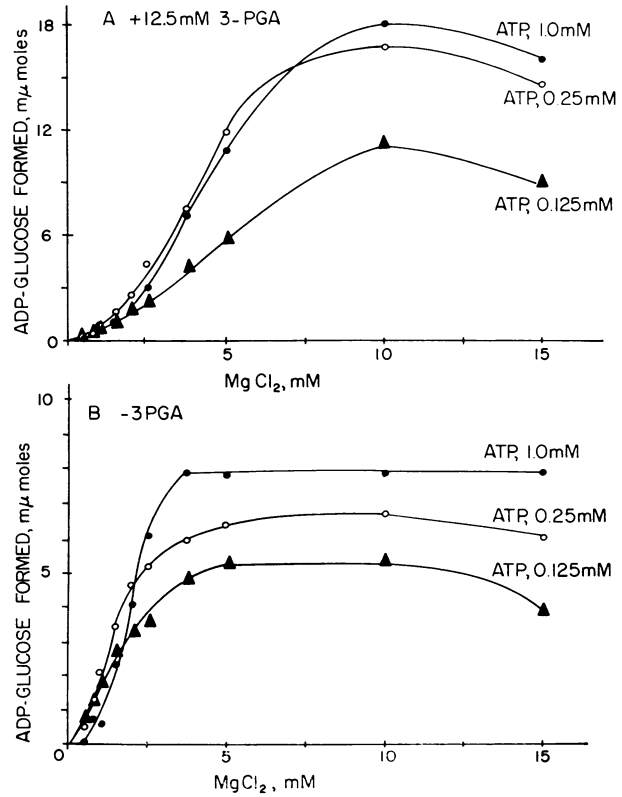


FIG. 4. Effect of $MgCl_2$ concentration on the rate of ADP-glucose synthesis catalyzed by embryo enzyme. The conditions of the experiment are those of assay B except the concentration of ATP and 3-PGA were varied as indicated.

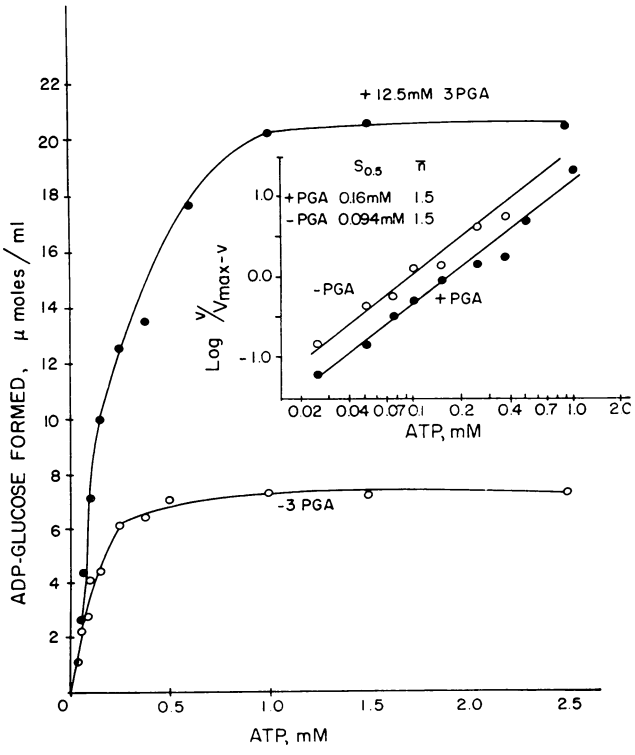


FIG. 5. Effect of ATP concentration on the rate of ADP-glucose synthesis in the presence and absence of 3-PGA. The conditions of the experiment are those of assay B. $S_{0.5}$ represents the concentration of substrate required for 50% of maximal velocity.

thesis of ADP-glucose. Varying the ATP concentration about 8-fold (from the concentration giving 50% of maximal velocity to saturation) did not greatly vary the amount of $Mg^{2+}$ required for maximal or half-maximal rates either in the presence or absence of 3-PGA. Thus, although 3-PGA increased the maximal velocity, it also increased slightly the concentration of $MgCl_2$ necessary for optimal activity.

Figure 5 shows the effect of 3-PGA on the ATP saturation curve. In the absence of the activator, 3-PGA, less ATP is required to saturate the enzyme than in its presence. The ATP saturation curve in the absence or presence of 12.5 mM 3-PGA appears to be sigmoidal in shape, and a plot of the data according to the Hill equation (inset, Fig. 5) gave an $\bar{n}$ value of 1.5 for both curves. The concentration of ATP required for 50% of maximal activity ($S_{0.5}$) is 0.16 mM in the presence of 3-PGA and 0.094 mM in the absence of 3-PGA.

Saturation curves for $\alpha$-glucose-1-P (Fig. 6) were hyperbolic and plots of the data according to the Hill equation gave $\bar{n}$ values of 1.0 in the absence of the activator and 0.98 in the presence of 12.5 mM 3-PGA. The $Km$ for $\alpha$-glucose-1-P was 0.034 mM in the absence of 3-PGA and 0.13 mM in the presence of 12.5 mM 3-PGA. The maximal velocity of ADP-glucose synthesis carried out by the ammonium sulfate fraction of the embryo ADP-glucose pyrophosphorylase was 5.6 $\mu$moles per mg protein in 30 min at 37 C in the presence of 12.5 mM 3-PGA and 1.75 $\mu$moles per mg protein in 30 min at 37 C in the absence of 3-PGA.

Although there was a stimulation of maximal velocity of 3-fold by 3-PGA, the activator caused an apparent decrease in affinity of the enzyme for its substrates ATP, glucose-1-P, and for $Mg^{2+}$; the greatest effect being a 4-fold increase in $Km$ for glucose-1-P. This effect is in contrast to what had been observed previously for the maize endosperm enzyme where 3-PGA not only increased maximal velocity but also increased the apparent affinity of the enzyme for both ATP and glucose-1-P (4).

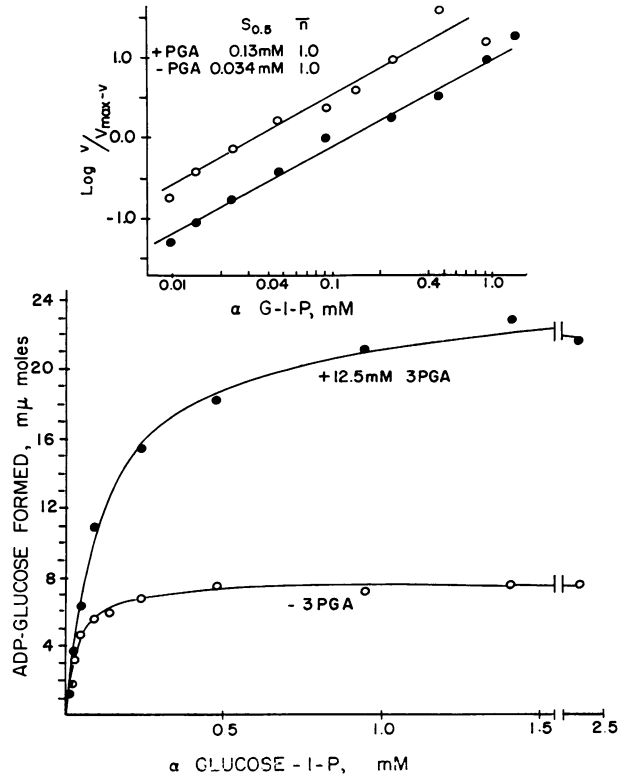


FIG. 6. Effect of $\alpha$-glucose-1-P concentration on the rate of ADP-glucose synthesis in the presence and absence of 3-PGA. The conditions of the experiment are those of assay B.

**Inhibition of ADP-Glucose Synthesis by Phosphate.** Figure 7 shows the inhibition of ADP-glucose synthesis catalyzed by the maize embryo enzyme by orthophosphate. The embryo enzyme is inhibited 50% by 0.32 mM phosphate ($I_{0.5}$) in the absence of 3-PGA and in the presence of 12.5 mM 3-PGA is inhibited 50% by 0.24 mM phosphate. Thus, sensitivity of the enzyme to inhibition by phosphate is little affected by the presence of 3-PGA. The maize embryo enzyme differs from the endosperm enzyme with respect to phosphate inhibition in two ways. First, the embryo enzyme is more sensitive to phosphate inhibition in the absence of 3-PGA than is the endosperm enzyme. Whereas 3.0 mM phosphate is required for 50% inhibition of the endosperm

Table II. *Some Kinetic Parameters of the $Sh_2$ Maize Embryo ADP-Glucose Pyrophosphorylase*

$S_{0.5}$, $A_{0.5}$, and $I_{0.5}$ represent concentrations of substrate, activator, or inhibitor required to give 50% of the maximal effect (velocity, inhibition, or activation). 3-PGA when present was used at a concentration of 12 mM. The apparent order of reaction obtained from the Hill equation is $\bar{n}$.

| Substrate/Effector | Condition | $S_{0.5}$ | $I_{0.5}$ | $A_{0.5}$ | $\bar{n}$ |
|---|---|---|---|---|---|
| | | mM | | | |
| ATP | +3-PGA | 0.16 | | | 1.4 |
| | −3-PGA | 0.14 | | | 1.3 |
| Phosphate | +3-PGA | | 0.24 | | 1.0 |
| | −3-PGA | | 0.24 | | 0.9 |
| 3-PGA | | | | 3.6 | 1.8 |

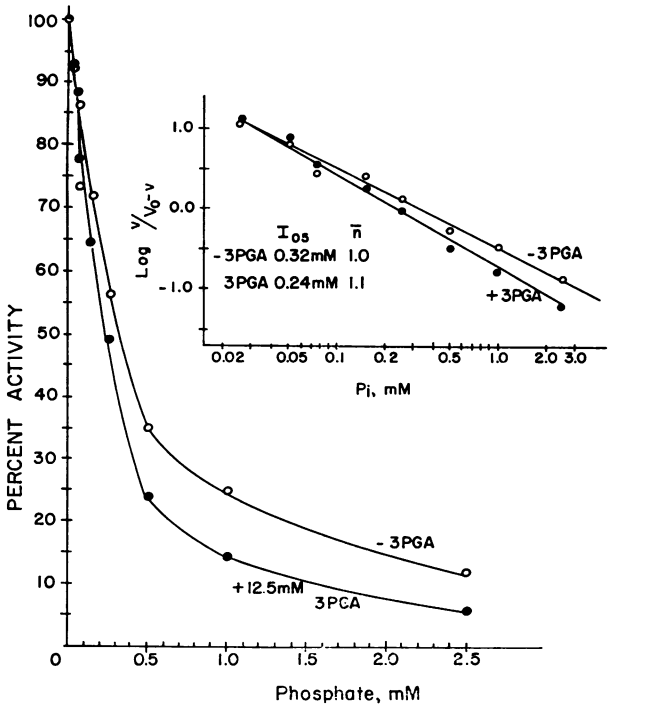


FIG. 7. Inhibition of ADP-glucose synthesis by phosphate in the presence and absence of 3-PGA. The conditions of the experiment are those of assay B and $I_{0.5}$ represents the concentration of inhibitor required for 50% inhibition under the conditions of the experiment. $V_0$ represents the velocity of the reaction in the absence of inhibitor. In the presence of 12.5 mM 3-PGA, 100% activity represents 12.6 m$\mu$moles of ADP-glucose synthesized in 30 min while in the absence of 3-PGA, 100% activity equals 9.4 m$\mu$moles of ADP-glucose synthesized in 30 min. Twice as much enzyme was used in the reaction mixtures having no activator.

ADP-glucose pyrophosphorylase (4), only 0.32 mM phosphate is required for 50% inhibition of the embryo enzyme. Second, the presence of 3-PGA has little effect on the concentration of phosphate required to inhibit the embryo ADP-glucose pyrophosphorylase; in fact a slightly smaller concentration of phosphate, 0.24 mM is required to give 50% inhibition. In contrast, it has been reported that in the presence of 3-PGA the endosperm enzyme becomes more resistant to phosphate inhibition. A higher concentration of phosphate (~10 mM) is required for 50% inhibition of the maize endosperm ADP-glucose pyrophosphorylase in the presence of 3-PGA (4).

Preliminary studies indicate that phosphate inhibition of the embryo enzyme is only slightly reversed by higher concentrations of 3-PGA (>20 mM) and is not reversed by higher concentrations of either ATP, $Mg^{2+}$, or glucose-1-P. Potassium sulfate and KCl at concentrations of 5 mM did not cause significant inhibition of the embryo enzyme. AMP at 1 mM caused 20% inhibition and at 10 mM caused 40% inhibition. ADP at 1 mM caused 44% inhibition and at 10 mM caused 95% inhibition.

**Properties of the Embryo ADP-Glucose Pyrophosphorylase Isolated from $Sh_2$ Maize Seeds.** The ADP-glucose pyrophosphorylase associated with the embryo tissue from $Sh_2$ maize seeds was found to have the same properties as the enzyme isolated from DeKalb 805 starchy maize embryos with respect to heat stability. About 85% of the ADP-glucose pyrophosphorylase activity in the $Sh_2$ maize embryos remained after heat treatment at 65 C for 5 min. Table II summarizes various kinetic parameters obtained with the embryo ADP-glucose pyrophosphorylase from $Sh_2$ seeds. The saturation curves for 3-PGA activation, for phosphate inhibition (± 3-PGA), and for ATP (± 3-PGA) were essentially the same as those observed for the embryo ADP-glucose pyrophosphorylase isolated from DeKalb 805 seeds.

## DISCUSSION

The above experiments strongly suggest the presence in embryo tissue of starchy maize of an ADP-glucose pyrophosphorylase distinct from the ADP-glucose pyrophosphorylase present in the endosperm. The embryo enzyme is more heat stable and is more sensitive to phosphate inhibition than its endosperm counterpart. Although both enzymes are activated by 3-PGA, the effects of 3-PGA on the substrate affinities for the enzymes are different. The presence of 3-PGA increases the apparent affinity of the endosperm ADP-glucose pyrophosphorylase for its substrates ATP and $\alpha$-glucose-1-P (4) while 3-PGA decreases the apparent affinity of the embryo enzyme for ATP and $\alpha$-glucose-1-P.

3-PGA can reverse to a great extent the inhibition caused by phosphate of the endosperm enzyme (4), but there is little effect of 3-PGA on the inhibition by phosphate on the embryo ADP-glucose pyrophosphorylase (Fig. 7; unpublished results). Other differences between the embryo and endosperm ADP-glucose pyrophosphorylases are observed in the specificity of activation with respect to fructose-6-P and ribose-5-P, in the pH range where maximum activation by 3-PGA occurs (Fig. 2), and in the shape of the 3-PGA activation curve (Fig. 3).

It had been shown previously that the level of ADP-glucose pyrophosphorylase present in $Sh_2$ mutant maize endosperm was greatly reduced while the level of enzyme in the $Sh_2$ mutant embryos were at the same levels as found in the normal starchy maize embryo (5). This finding would be consistent with the above kinetic studies and would suggest that in $Sh_2$ and DeKalb 805 seeds that there are two different structural genes coding for ADP-glucose pyrophosphorylase; one gene would code for the endosperm enzyme and the other would code for the enzyme present in the embryo tissue. The mutation in $Sh_2$ would then only affect the ADP-glucose pyrophosphorylase activity present in the endosperm.

It is interesting to note that Akatsuka and Nelson (1) reported the presence of two forms of ADP-glucose:starch glucosyl transferase in nonwaxy maize seeds. The two forms were distinguished by various kinetic parameters and by different heat stabilities at 60 C in the presence or absence of EDTA and KCl. One form was associated with the embryo tissue and the other form was present in endosperm tissue. Tsai and Nelson (10) have also found a specific phosphorylase associated with the embryo. It therefore appears that maize embryo tissue has distinct and different forms of the enzymes required for the synthesis and breakdown of starch.

At present there is no apparent physiological significance of 3-PGA activation of the embryo ADP-glucose pyrophosphorylase. Although there is an actual increase of maximal velocity in the presence of 3-PGA, there is also a decrease in the affinities of the substrates for the enzyme. This effect is opposite to what has been observed for the ADP-glucose pyrophosphorylases present in spinach leaf tissue (6), *Chlorella* (8), and in maize endosperm (4), where 3-PGA not only increases maximal velocity but also increases the apparent affinities of the substrates for the enzyme. It would be of importance to determine the intracellular levels of the pertinent metabolites in embryo tissue before a rationalization of the effect of 3-PGA on the embryo ADP-glucose pyrophosphorylase can be made.

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