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Phosphorylases I and II of Maize Endosperm[1, 2, 3]

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Abstract. Two phosphorylases have been found in the endosperm of *Zea mays*. Phosphorylase I is found through all stages of endosperm development and seed germination investigated. The other enzyme, phosphorylase II appears only at the stage of rapid starch biosynthesis and is not found during germination. At 22 days after pollination, the activity of phosphorylase II is 10 times that of phosphorylase I. These 2 phosphorylases are separable by column chromatography and behave differently in several respects.

Phosphorylase I cannot utilize maltose as a primer while phosphorylase II does so readily. Furthermore, phosphorylase II can synthesize an amylose-like polymer from a "primer free" system after a lag phase.

Phosphorylase II is inhibited severely at pH 5.8 by ATP, GTP, ADP, and GDP, and less drastically by UTP, CTP, UDP and CDP. Phosphorylase I is somewhat inhibited by purine nucleotides but not by pyrimidine nucleotides. In all cases, the inhibition is pH-dependent. Phosphorylase I is inhibited competitively by ATP while phosphorylase II is inhibited non-competitively.

Phosphorylase II is markedly stimulated by 10 mM Mg^{2+} and by 2 mM ethylenediamine tetraacetic acid while phosphorylase I is relatively little affected.

Phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase) was first discovered in liver tissue (2) to catalyze a reversible reaction between glucose-1-phosphate (glucose-1-P) and glycogen.

Glucose-1-P + Glycogen $(G)_n \rightleftarrows$ Glycogen $(G)n+1$ + Pi. Soon after this discovery, the same type of reaction was demonstrated in tissues of higher plants (8, 9). It was then assumed that phosphorylase might be playing an important role in glycogen and starch biosynthesis. However, in 1957 another enzyme, uridine diphosphate glucose-glycogen transglucosylase (UDPG-glycogen transglucosylase), was found in liver tissue (14). It used uridine diphosphate glucose (UDPG) as a substrate and transferred the glucose moiety into glycogen. Subsequently, a similar enzyme was found bound to the starch granules of higher plants (4, 15), but this enzyme preferentially used adenosine diphosphate glucose (ADPG) as a substrate (20, 22).

UDPG (ADPG) + Primer (G)n ——— UDP (ADP) + Product $(G)_{n+1}$

The energy level of the hydrolysis of the glucosidic phosphate bond ($\Delta F^\circ = -7600$ cal) of UDPG is greater than that of the phosphate ester linkage ($\Delta F^\circ = -4800$ cal) of the glucose-1-P with respect to the α-1,4 linkage ($\Delta F^\circ = -4300$ cal) in the polysaccharide. Also the overall reaction in the transglucosylase system (K′ eq=250) is more favorable for synthesis in comparison to that of the phosphorylase system (K′ eq=3) at pH 7.5 (10). The most direct evidence that most starch biosynthesis occurs via a transglucosylase system comes from studies of a starch-deficient maize mutant, *shrunken-2* (sh_2), that lacks adenosine diphosphate glucose pyrophosphorylase (ADPG-pyrophosphorylase) activity (26). Starch synthesis is substantially reduced, probably because the substrate, ADPG, for ADPG-starch transglucosylases is limiting.

The above evidence suggests that the starch synthesis is predominantly via transglucosylases and rules out the possibility that the major portion of starch synthesis proceeds by way of starch phosphorylase. Other arguments were earlier raised against the participation of starch phosphorylase in biosynthesis. The high Pi/glucose-1-P ratio in the living bark of black locust (6) suggested that starch phosphorylase was mainly responsible for starch degradation in that tissue.

In spite of these arguments against starch phosphorylase as a major enzyme for starch biosynthesis, there are indications that transglucosylase reactions may not be wholly responsible for synthesis. Since transglucosylases require a primer of short chain length, the initiation of starch synthesis remains a problem. Recently, studies on muscle phosphorylase and potato phosphorylase showed that the *de novo* synthesis of amylose can proceed in the complete absence of primer (11) which suggests that phosphorylase could be of special importance in the early stage of polysaccharide biosynthesis.

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Also studies of carbohydrate storage products in starch-forming mutants of maize (3, 12, 19) have been interpreted as indicating that there is more than one pathway of starch synthesis. Therefore, phosphorylase activity in maize endosperm has been examined in detail.

Materials and Methods

Substrates and Reagents. Sodium salts of UTP, GTP, CTP and ADP were purchased from Pabst Laboratories. Disodium salts of glucose-1-P, glucose-6-P, UDP, GDP, and dipotassium salts of ATP, CDP, and sodium salts of 3-P-D-glyceric acid, pyruvic acid, and tricyclohexylammonium salt of 2-P-enolpyruvic, and AMP, Cellex D (DEAE: diethylaminoethyl), 2-(N-morpholino)-ethanesulfonic acid·H_2O (MES), reduced glutathione (GSH), and amylopectin were purchased from Calbiochem. The tetracyclohexylammonium salt of fructose-1,6-diP and the barium salt of fructose-6-P were purchased from Boehringer and Sons, Germany. Maltose was purchased from Nutritional Biochem. and glucose from Baker Company. The uniformly labeled glucose-^{14}C-1-P (specific activity 47 mc/mmole) was obtained from Calbiochem.

Preparation of Enzymes. Samples of the normal (non-mutant maize, *Zea mays* L.) hybrid, B37XB14, were collected 22 days after controlled self-pollination by freezing the entire ear on dry ice. The samples were stored at −20° until processing.

As the initial step in processing, the kernels were cut from the cob. The embryo and the pericarp or seed coat were removed from the kernel, leaving the endosperm with a thin layer of closely adhering tissue from the female parent.

A mixture of equal parts of endosperms and chilled 0.01 M tris-maleate buffer (pH 7.0), was homogenized for 3 minutes in a blender, strained through 2 layers of cheese cloth, and centrifuged for 20 minutes at 29,000 × *g*. From the supernatant, the fraction precipitating at 25 to 45 % $(NH_4)_2SO_4$ saturation was collected by centrifugation for 20 minutes at 18,800 × *g*, suspended in chilled 0.01 M tris-maleate buffer (pH 7.0), and dialyzed against the same buffer for 8 hours at 4°.

About 25 mg of protein/2 ml [measured by the method of Lowry *et al.* (16)] was then added to a DEAE-cellulose column (1 cm × 20 cm) equilibrated with 0.01 M tris-maleate buffer at pH 7.0. The proteins were eluted with a linear 0 to 1 M NaCl gradient, buffered with the tris-maleate buffer, and 5 ml fractions were collected. The enzyme fractions were again dialyzed against 0.01 M tris-maleate buffer (pH 7.0), for 8 hours at 4° to remove the salt.

Survey of Enzymic Activity by Measuring the Incorporation of Glucose-^{14}C into an Amylopectin Primer. The reaction mixture contained 10 μmoles of MES buffer and 0.2 μmole of uniformly labeled glucose-^{14}C-1-P (26,000–32,000 counts/min), 5 mg of amylopectin and 10 μliters of enzyme solution containing about 3 μg of protein in a total reaction volume of 50 μliters for the phosphorylase II system and 20 μliters of enzyme fraction containing about 7 μg of protein in a total reaction volume of 60 μliters for the phosphorylase I system. Incubation was at 37° in a water bath for 15 to 60 minutes.

The enzymic reaction was terminated by the addition of 0.5 ml of 0.1 N NaOH followed by the precipitation of amylopectin with a final concentration of 75 % methanol. The pellet was collected by centrifugation and washed 3 times with 0.1 N NaOH followed by methanol precipitation and centrifugation. After resuspension in 0.1 N NaOH, the entire amount of suspended material was then distributed over a ringed planchet, 1.25 inch in diameter, and evaporated under an infrared lamp before counting in a gas flow counter (Nuclear-Chicago). All data presented have been corrected by control values from reaction mixtures that did not contain enzyme.

Measurement of Enzymic Activity with Iodine Staining. The reaction mixture contained 20 μmoles of tris-maleate buffer (pH 7.0), 6 μmoles of glucose-1-P and 0.25 % of maltose, 60 μg of protein/0.2 ml for the phosphorylase II or 70 μg of protein/0.2 ml for the phosphorylase I system in a total reaction volume of 0.4 ml. After the reaction was terminated in a boiling water bath for 30 seconds, the reaction mixture was centrifuged. To the clear supernatant, 0.1 ml of 0.2 % iodine in 1 % KI solution was added. After the addition of iodine solution, a dilution of 1 to 10 with water was made before reading in a Spectronic 20 at 660 mμ.

Measurement of Enzymic Activity from Germinating Seeds. Mature seeds of the normal maize (*Zea mays* L.) hybrid, B37XB14, were placed in water and aerated overnight, then germinated in sand for 3 days. The enzyme extraction and the method of assay were based on the procedure described under Preparation of Enzymes.

Measurement of Enzymic Activity at Different Stages of Endosperm Development. The whole ears of the normal hybrid, B37XB14, were collected 6, 12, 16, 22, and 28 days after controlled self-pollination as described in the Preparation of Enzymes.

The initial processing step was essentially similar to the procedure described in the Preparation of Enzymes, but for those materials collected 6 and 12 days after pollination, the embryo was not separated from the endosperm because of its small size. Only the seed coat was removed.

The enzyme extraction was essentially similar to the procedure described in the Preparation of Enzymes except the $(NH_4)_2SO_4$ precipitation was omitted. The method of assay was based on the procedure described in the Survey of Enzymic Activity by Measuring the Incorporation of Glu-

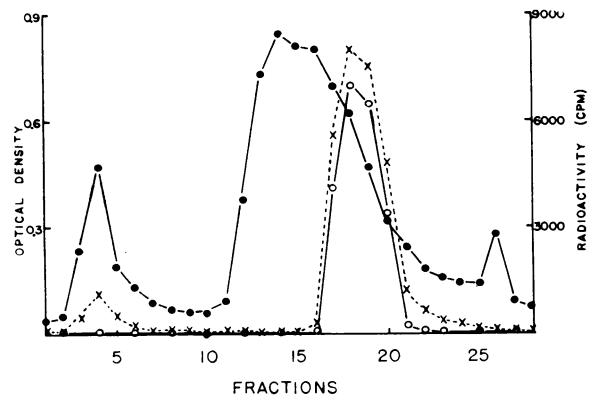

FIG. 1. Phosphorylase activity in the maize endosperm. For the tracer assay, reaction mixtures contained 10 μmoles of MES buffer, pH 5.8, 0.2 μmole of glucose-^{14}C-1-P (32,000 cpm), 5 mg of amylopectin and 30 μliters of enzyme fraction in a total reaction volume of 60 μliters. Incubation was 30 minutes. For the iodine staining technique, reaction mixtures contained 20 μmoles of tris-maleate buffer, pH 7.0, 6 μmoles of glucose 1-P, 0.25 % of maltose and 0.2 ml of enzyme fraction in a total reaction volume of 0.4 ml. Incubation was 45 minutes at 37°. ●—●—● Protein profile of DEAE-cellulose fractionation (measured by Lowry method). X-X-X Activity measured by the incorporation of glucose-^{14}C into amylopectin primer. ○—○—○ Activity measured by iodine staining technique using a maltose primer.

cose-^{14}C into an Amylopectin Primer. Activity was expressed on a per endosperm basis.

Results

Phosphorylase Activities in the Maize Endosperm. Figure 1 indicates that the preparation from the developing endosperm 22 days after pollination gives 2 peaks of phosphorylase activity that are physically separable by DEAE-cellulose column chromatography. Both enzymes are capable of transfering the glucose moiety from glucose-1-P into starch. Only the later peak enzyme is capable of synthesizing a blue-staining polymer when maltose is the primer. The early peak activity (maximum activity at fraction 4), after DEAE-cellulose column fractionation, is the enzyme designated as phosphorylase I while the later peak (maximum activity at fraction 18) is phosphorylase II. Phosphorylase II activity is about 10 times that of phosphorylase I on a per endosperm basis.

Table I. *The Specific Activity of Phosphorylases I and II*

Reaction mixtures contained 0.2 μmole of glucose-^{14}C-1-P (28,000 cpm), 10 μmoles of MES buffer, pH 5.8, 5 mg of amylopectin and 10 μliters of enzyme solution in a total reaction volume of 50 μliters. Incubation was at 37° for 30 minutes.

Enzyme	Specific activity (μmole of glucose-^{14}C × 10^{-4} incorporated/μg of protein/30 mins)
Dialyzed homogenate	0.33
Phosphorylase I	8.62
Phosphorylase II	80.40

The specific activity (μmoles of glucose-^{14}C × 10^{-4} incorporated/μg of protein/30 mins) for the phosphorylase I and II preparations is 8.62 and 80.40 respectively at pH 5.8 (table I). The activity of both phosphorylases was found to be linearly proportional to the concentration of protein assayed.

The Effect of Cations, EDTA, and GSH on Phosphorylase I and Phosphorylase II. Table II shows that at 4 mM glucose-1-P, 10 mM Mg^{2+} and Mn^{2+} stimulate phosphorylase I activity by 50 % while phosphorylase II is stimulated more than 300 %. Phosphorylase I is inhibited 60 % by 2 mM Zn^{2+} and 50 % by 2 mM Fe^{2+} while 2 mM Fe^{2+} has no effect on phosphorylase II. Phosphorylase II is completely inhibited by 2 mM Zn^{2+}. Both enzymes are strongly inhibited by 2 mM concentrations of heavy metal ions. EDTA (2 mM) has little effect on phosphorylase I, but it stimulates phosphorylase II more than 300 %. Phosphorylase I is not affected by 5 mM GSH, but phosphorylase II is completely inactivated.

Table II. *The Effect of Cations, EDTA and Reduced Glutathione on Phosphorylases I and II*

Reaction mixtures containing 0.2 μmole of glucose-^{14}C-1-P (26,000 cpm) 10 μliters of additive, 10 μmoles of MES buffer, pH 5.8, 5 mg of amylopectin and enzyme as given under Methods, were incubated for 15 minutes for phosphorylase II and 45 minutes for phosphorylase I.

Additives	Amount of additive	Radioactivity: Phosphorylase II	Radioactivity: Phosphorylase I
	μmole	*cpm*	*cpm*
None		310	629
Mg^{2+}	0.5	955	808
Mn^{2+}	0.5	997	972
Fe^{2+}	0.1	311	327
Zn^{2+}	"	0	230
Cu^{2+}	"	10	6
Ag^{+}	"	18	15
Hg^{2+}	"	22	18
EDTA	"	1020	701
GSH[1]	"	10	504

[1] Enzyme was preincubated with GSH for 30 minutes before the addition of glucose-1-P.

The Effect of Nucleotides and Glycolytic Intermediates on the Activity of Phosphorylase I and Phosphorylase II. Phosphorylase I is inhibited about 45 % at pH 5.8 by purine nucleoside diphosphates and triphosphates, but is not affected by

Table III. *The Effect of Nucleotides and Glycolytic Intermediates*

Reaction mixtures containing 0.2 μmole of glucose-^{14}C-1-P (32,000 cpm), 10 μliters of additive, 10 μmoles of MES buffer in a final volume of 50 μliters, and enzyme as in Methods were incubated for 30 minutes for phosphorylase II and 60 minutes for phosphorylase I.

Additives	Amount of additive	Radioactivity Phosphorylase II pH 5.8	Phosphorylase II pH 7.0[3]	Phosphorylase I pH 5.8
	μmole	*cpm*	*cpm*	*cpm*
None		1106	2532	780
ATP	0.20	416	2701	427
UTP	"	707	2515	713
GTP	"	115	2636	478
CTP	"	689	2535	757
ADP	0.25	365	2614	404
UDP	"	759		736
GDP	"	322		343
CDP	"	747		748
5′-AMP	0.10	820		792
Glucose-6-P	0.20	1170		755
Fructose-6-P	"	1114		789
Fructose-1,6-diP	"	1108		768
3-Phospho-D-glyceric acid	"	1100		780
P-enolpyruvate	0.20	1053		764
Pyruvate	"	1256		711
Mg^{2+}	0.5	3533		
Mn^{2+}	"	3685		
Mg^{2+} + ATP[1]		640		
Mn^{2+} + ATP[1]		740		
ATP + Mg^{2+} [2]		300		
ATP + Mn^{2+} [2]		270		

[1] Enzyme preincubated with cation for 15 minutes then ATP and glucose-1-P were added.
[2] Enzyme preincubated with ATP for 15 minutes then the cation and glucose-1-P were added.
[3] Data obtained from different enzyme preparations.

2 mM AMP (table III). Phosphorylase II is severely inhibited by purine nucleoside diphosphates and triphosphates with GTP being most effective. AMP gives about 25 % inhibition. Pyrimidine nucleotides inhibit approximately 35 %. The glycolytic intermediates tested, glucose-6-P, fructose-6-P, fructose-1,6-diP, 3-P-glycerate, 2-P-enolpyruvate, and pyruvate, have no effect on either enzyme.

The pH-dependent ATP Inhibition of Phosphorylase I and Phosphorylase II. Figure 2 shows that the pH optimum for phosphorylase I is 5.8 and the enzyme activity is about 35 % of maximum at pH 5.0. At pH values higher than 7.0, ATP has no effect on this enzyme. However, when the pH value is lowered to 6.5 inhibition is noted.

For phosphorylase II, figure 3 shows that the pH optimum is 5.9, and activity is completely lost at pH 5.0. ATP has no effect on this enzyme at pH values higher than 6.0. However, when the pH value is lowered to 5.9 a pronounced inhibition is observed. The phosphorylase II activity is completely inhibited by ATP at pH 5.5.

The Km Values for Phosphorylase I and Phosphorylase II. The Km values with glucose-1-P as a substrate and amylopectin (5 mg/assay tube) as a primer, with the velocity (cpm/unit time) plotted against the higher range of substrate concentration, were 3.3 mM for phosphorylase I and 4 mM for

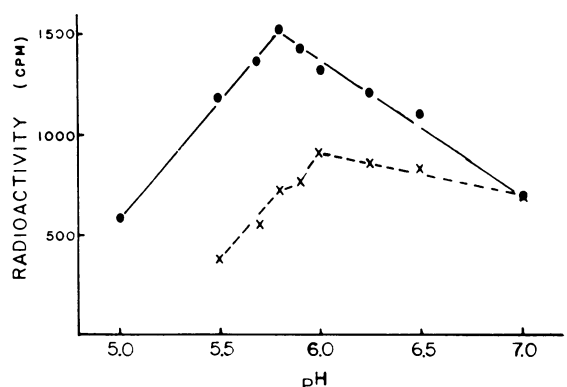

FIG. 2. pH-dependent ATP effect on phosphorylase I. Reaction mixtures contained 10 μmoles of MES buffer, 0.2 μmole of glucose-^{14}C-1-P (32,000 cpm), 5 mg of amylopectin and 7 μg of protein/20 μliters in a total reaction volume of 60 μliters. Incubation was 60 minutes at 37°. ●—●—● Reaction mixture contained no ATP. X-X-X Reaction mixture contained 0.2 μmole ATP.

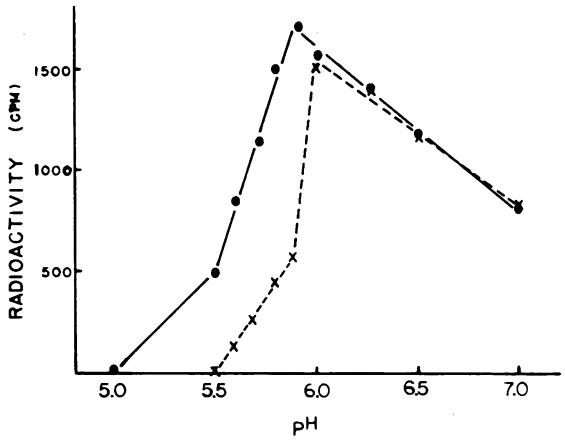

FIG. 3. pH-dependent ATP effect on phosphorylase II. Reaction mixtures contained 10 μmoles of MES buffer, 0.2 μmole of glucose-^{14}C-1-P (32,000 cpm), 5 mg of amylopectin and 3 μg of protein/10 μliters in a total reaction volume of 50 μliters. Incubation was 30 minutes at 37°. ●—●—● Reaction mixture contained no ATP. X-X-X Reaction mixture contained 0.2 μmole of ATP.

phosphorylase II at pH 5.8. As indicated in figure 4, for phosphorylase I in the presence of the inhibitor (ATP), the Vmax remains unchanged, but the Km is increased. This is characteristic of competitive inhibition. For phosphorylase II, on the other hand, the Vmax is decreased by ATP, but the Km value remains the same whether ATP is present or absent (fig 5). This indicates that phosphorylase II is non-competitively inhibited by ATP.

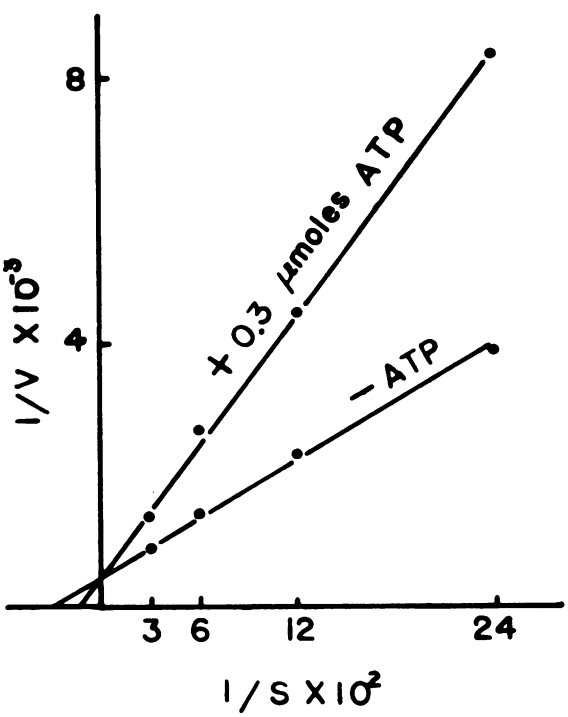

FIG. 4. Km determination for phosphorylase I. Reaction mixtures contained 10 μmoles of MES buffer, pH 5.8, 5 mg of amylopectin and 7 μg of protein/20 μliters in a total reaction volume of 60 μliters. Incubation was 60 minutes at 37°. V=cpm/60 min.

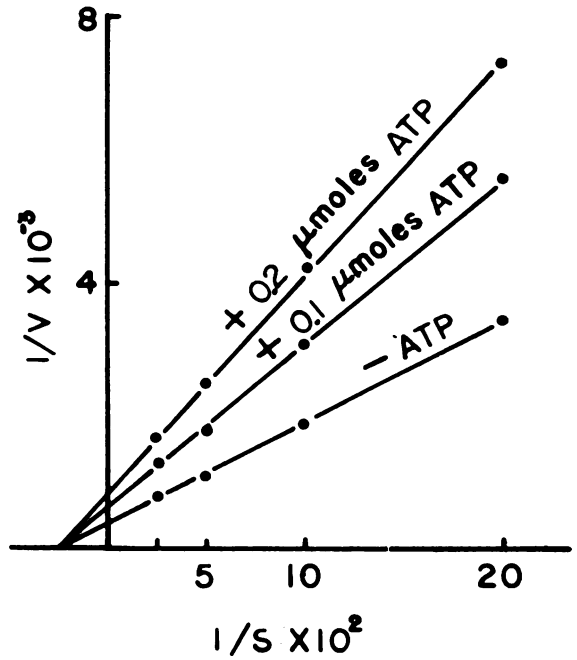

FIG. 5. Km determination for phosphorylase II. Reaction mixtures contained 10 μmoles of MES buffer, pH 5.8, 5 mg of amylopectin and 3 μg of protein/10 μliters in a total reaction volume of 50 μliters. Incubation was 30 minutes at 37°. V=cpm/30 min.

The Effect of ATP and Substrate Concentration on Phosphorylases I and II. At a glucose-1-P concentration of 3.3 mM, phosphorylase I activity is inhibited by higher concentrations of ATP. However, at low concentrations of ATP the activity is stimulated as shown in figure 6. With phosphorylase II, when the concentration of glucose-1-P is held constant (4 mM), the concentration of ATP varied, and the results plotted as percent inhibition against the concentration of ATP, the resulting curve approaches a sigmoidal curve in form (fig 7). This raises the question as to whether phosphorylase II is an allosteric protein on which ATP acts as an effector (17). When the activity is plotted against substrate concentration for phosphorylase II (fig 8), the curve is not as sigmoidal in form as that relating activity to the concentration of ATP.

Primer Requirements for Phosphorylases I and II. Figure 1 shows that only phosphorylase II has the ability to synthesize an amylose-like polymer with maltose as a primer. Phosphorylase I shows no such activity even when incubated for 12 hours. The inability to detect the synthesis of an amylose-

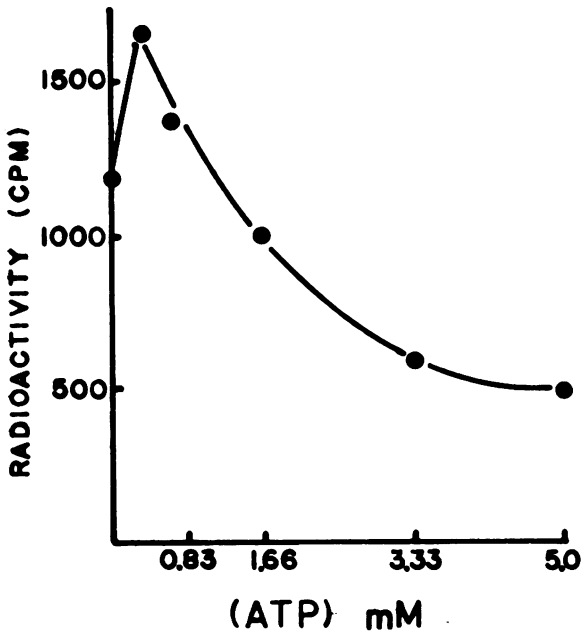

FIG. 6. The effect of ATP concentration on phosphorylase I activity. Reaction mixtures contained 10 μmoles of MES buffer, pH 5.8, 10 μliters of ATP solution, 0.2 μmole of glucose-^{14}C-1-P (32,000 cpm), 5 mg of amylopectin and 7 μg of protein/20 μliters in a total reaction volume of 60 μliters. Incubation was 60 minutes, 37°.

like polymer by phosphorylase I is not due to the presence of other starch degradative enzymes. A mixture of both phosphorylase I (fraction 4) and phosphorylase II (fraction 21) shows the phosphorylase II activity is not affected as measured either by the iodine staining technique or incorporation (table IV). Besides using maltose as primer, phosphorylase II can synthesize an amylose-like polymer from a "primer free" system (fig 9).

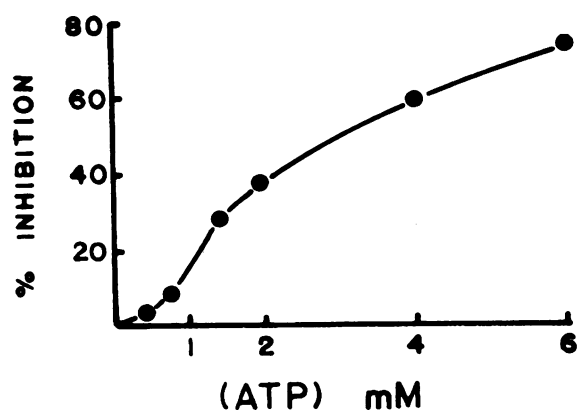

FIG. 7. The effect of ATP concentration on phosphorylase II activity. Reaction mixtures contained 10 μmoles of MES buffer, (pH 5.8), 10 μliters of ATP solution, 0.2 μmoles of glucose-^{14}C-1-P (32,000 cpm), 5 mg of amylopectin and 3 μg of protein/10 μliters in a total reaction volume of 50 μliters. Incubation was 30 minutes, 37°.

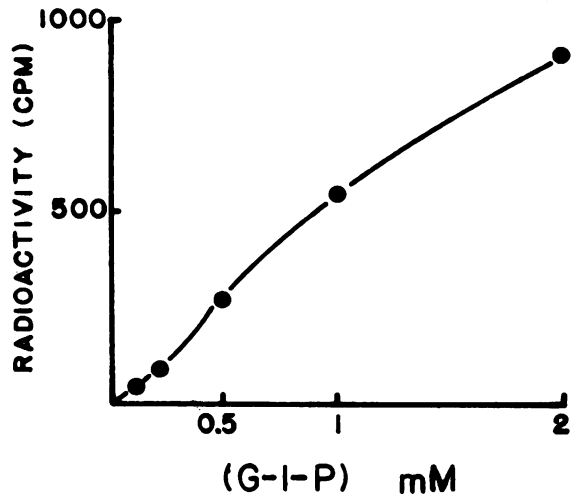

FIG. 8. The effect of G-1-P concentration on phosphorylase II. Reaction mixtures contained 10 μmoles of MES buffer, (pH 5.8), 10 μliters of glucose-^{14}C-1-P (2000–36,000 cpm) and 3 μg of protein/10 μliters in a total reaction volume of 50 μliters. Incubation was 30 minutes, 37°.

Phosphorylase Activity during Endosperm Development. Phosphorylase activity is determined by measuring the whole peak of activity after separation on the column. Since the purpose here is to study the development of the endosperm, the enzymic activity is expressed as μmoles of glucose-^{14}C × 10^{-1} incorporated per endosperm into an amylopectin

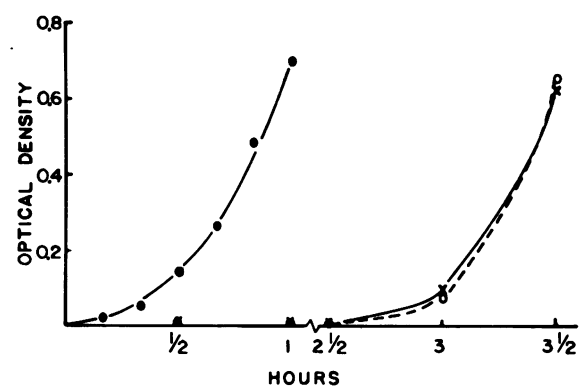

FIG. 9. Priming activity for phosphorylase II. Reaction mixtures contained 20 μmoles of tris-maleate buffer, pH 7.0, 6 μmoles of glucose-1-P, 0.25 % maltose or 0.125 % glucose as primers, and 0.2 ml of enzyme fraction in a total reaction volume of 0.4 ml. After termination of the reaction, the mixtures were reacted with a KI • I_2 stain. ●—●—● System contained 0.25 % maltose as a primer. X—X—X System contained 0.125 % glucose as a primer. ○-○-○ System contained no primer.

Table IV. *The Activity of Enzyme Mixtures*

The reaction mixtures for incorporation contained 0.2 μmole of glucose-^{14}C-1-P (26,000 cpm) 10 μmoles of MES buffer, pH 5.8 and enzyme solution (for phosphorylase I, fraction 4 was used, and for phosphorylase II, fraction 21 was used) were incubated for 30 minutes with amylopectin as a primer. When activity was measured by the production of a substance staining with iodine, incubation was 90 minutes with maltose as a primer.

Enzymes	Amount of enzyme added	Activity: Incorporation amylopectin primer	Activity: OD maltose primer
		cpm	
Phosphorylase I	10 μliters fraction 4 + 10 μliters H_2O	359	0.0
Phosphorylase II	10 μliters fraction 21 + 10 μliters H_2O	379	0.300
Phosphorylase I + II	10 μliters fraction 4 + 10 μliters fraction 21	689	0.300

primer. Figure 10 shows that phosphorylase I activity increases from 6 days to 28 days after pollination. The activity of phosphorylase II is not detectable at 12 days, has appeared at 16 days, and gives maximum activity at 22 days after pollination. Then the activity declines.

Phosphorylase Activity during Seed Germination. Phosphorylase II activity is not detectable 3 days after germination was initiated. Figure 11 gives the protein and activity profiles of an extract made from the germinating seeds. The ability to transfer glucose from glucose-1-P to the amylopectin primer is found only in the position characteristic of phosphorylase I. No detectable activity is found in the fractions where phosphorylase II would be present. No direct comparison for the phosphorylase I activity between endosperm development and seed germination has been made.

Inhibition and Reactivation of Phosphorylase II. Activity after ATP inhibition can be partially recovered by the readjustment of the reaction mixture to a higher pH range. Figure 12 shows that the addition of ATP during incubation at pH 5.5 shuts off the incorporation of glucose-^{14}C into the amylopectin primer very rapidly. However, with the readjustment of the inhibited system to above pH 6.5 the activity is partially restored. The inhibition of phosphorylase II activity by ATP is not relieved by either Mn^{2+} or Mg^{2+} at 10 mM concentrations (table III).

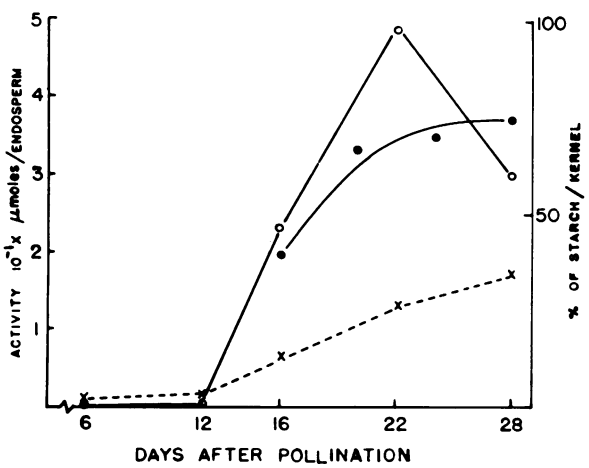

FIG. 10. Survey of phosphorylase activity during endosperm development. Reaction mixtures contained 10 μmoles of MES buffer, pH 5.8, 0.2 μmole of glucose-^{14}C-1-P (26,000 cpm), 5 mg of amylopectin and 10 μliters of enzyme fraction. Incubation was 45 minutes for phosphorylase I system and 15 minutes for phosphorylase II. ○—○—○ Phosphorylase II activity. X-X-X Phosphorylase I activity. ●—●—● Percent of starch.

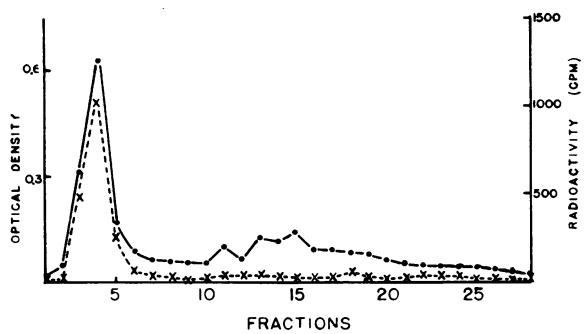

FIG. 11. Phosphorylase activity after 3 days of germination. Reaction mixtures contained 10 μmoles of MES buffer, (pH 5.8), 0.2 μmole of glucose-^{14}C-1-P (26,000 cpm), 5 mg of amylopectin, and 20 μliters of enzyme fraction. Incubation was 45 minutes. ●—●—● Protein profile of DEAE-cellulose column fractionation. X-X-X Radioactivity.

Discussion

Experimental data indicate that 2 kinds of phosphorylase activity are found in maize endosperm. The enzymes in these fractions behave differently

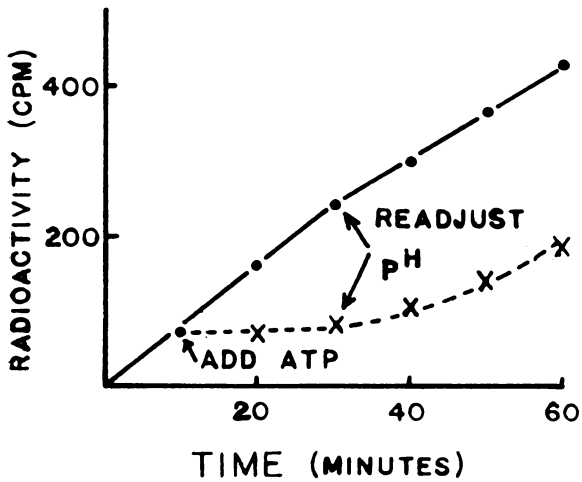

FIG. 12. Inhibition and reactivation of phosphorylase II. After 10 minutes of incubation, 0.2 μmole of ATP was added to a system containing 5 μmoles of MES buffer, pH 5.5, 0.2 μmole of glucose-^{14}C-1-P (26,000 cpm), 3 μg of protein/10 μliters enzyme solution and 5 mg of amylopectin. Twenty minutes after the addition of ATP, 20 μmoles of MES buffer (pH 7.0) were added to readjust the pH to above pH 6.5.

in several important aspects. Phosphorylase I and phosphorylase II have quite different responses to cations (table II).

Particularly interesting is the fact that both enzymes are found to be inhibited by purine nucleotides at pH 5.8. However, 2 mM AMP has no effect on phosphorylase I at the substrate (glucose-1-P) concentration of 4 mM, and 5 mM concentrations of pyrimidine nucleoside diphosphates and 4 mM triphosphates have little or no effect. Phosphorylase II is inhibited by 35 % by those levels of pyrimidine nucleoside diphosphates and triphosphates. The inhibition of both enzymes by ATP or GTP is found to be dependent on pH. For phosphorylase I, ATP inhibition occurs when the pH value is lowered to 6.5. For phosphorylase II, on the other hand, no inhibition can be observed when the pH value is higher than 6.0. If the pH value is lowered to 5.9, a pronounced inhibition by ATP is observed. This remarkable effect, by changing 0.1 unit of the pH scale, suggests that the protein conformation may be changed in such a way that the binding of ATP to the enzyme molecule can occur. The curve for the binding of the effector, ATP, to phosphorylase II appears sigmoid (fig 7). This suggests that phosphorylase II may be a regulatory enzyme (1). Since ATP, ADP, and 5′-AMP all inhibit phosphorylase II, when the pH is lowered to 5.9, and since the inhibition also can be partially reversed by raising the pH as shown in figure 12, pH could play an important role in regulating phosphorylase II activity *in vivo*. Phosphorylase II, however, is different from animal phosphorylase with respect to ATP inhibition. ATP inhibits competitively the activation of phosphorylase *b* by 5′-AMP. Thus, the ATP/AMP ratio is important as a control for phosphorylase *b* (18).

Since starch transglucosylases require a primer to start starch synthesis, it has been suggested that phosphorylase is important in primer synthesis (11). It has been shown that some starch phosphorylases are capable of synthesizing an amylose-like polymer from a "primer free" system (5,7). It is rather interesting to find that phosphorylase II can synthesize an amylose-like polymer from a "primer free" system while phosphorylase I is not active even with maltose as the primer. This leads to the question of the physiological significance of these 2 enzymes in maize endosperm.

Developmental studies indicate that phosphorylase I is constitutively present through all the stages of developing endosperm examined and is found during germination while phosphorylase II activity has not appeared. Phosphorylase I is competitively inhibited by ATP, and this inhibition may be a control mechanism of starch degradation during seed germination.

Phosphorylase II, on the other hand, can synthesize an amylose-like polymer from a "primer free" system, and one might suspect that this enzyme were responsible for primer synthesis. If this were the case, one should be able to see activity present at early stages of endosperm development. There is no detectable activity for phosphorylase II 12 days after pollination when starch is known to be present. Although phosphorylase I is present at early stages, this enzyme is not active with maltose as a primer. This evidence makes it doubtful that either phosphorylase I or II is responsible for primer synthesis in maize endosperm.

Phosphorylase II is only present at the period when starch synthesis is proceeding rapidly. This rapid synthesis spans the period from 16 to 28 days after pollination (3). Figure 10 shows the parallel increase of phosphorylase II activity and the formation of starch. One might wonder if this enzyme participates directly in starch biosynthesis. Starch phosphorylase has been considered as an unlikely candidate for starch synthesis because of high Pi/glucose-1-P ratio in many tissues. However, the studies on the developing pea seed indicated that the Pi/hexose monophosphate ratio decreased from the initial stage of 13 to 2 during the development (23). Further, much of the free phosphate may be sequestered in the vacuole (21).

Another argument against the participation of phosphorylase in starch synthesis came previously from studies on tobacco and potato leaf chloroplasts by Stocking (24). In this organelle, starch grains were found but no starch phosphorylase activity was detected by histochemical and biochemical techniques. However, the same investigator reported later that tobacco leaf chloroplasts isolated in non-

aqueous media contain at least 54% of the cell phosphorylase (25). Studies of tapioca and dahlia chloroplasts indicated that preparations from both plants had significant amounts of starch. There was no detectable activity for starch transglucosylase, only starch phosphorylase activity (27).

A starch-deficient maize mutant, *shrunken-2*, that lacks ADPG-pyrophosphorylase activity, synthesizes only 25 to 30% as much starch as normal maize (26). Since ADPG is the main substrate for both soluble and starch granule-bound transglucosylases, the relatively small amount of starch synthesis in this mutant may proceed, in part, by transfer of glucose from UDPG by soluble and starch granule-bound transglucosylase systems or from glucose-1-P by starch phosphorylase II.

One might ask if these 2 enzymes are interconvertible as are phosphorylase *a* and phosphorylase *b* in the animal tissue. No direct study has yet been made of this particular point. However, the studies of purified potato phosphorylase showed that potato phosphorylase could not be inactivated by a specific phosphatase (PR enzyme) which converts phosphorylase *a* to *b*. No phosphorylation could be detected by phosphorylase *b* kinase which converts phosphorylase *b* to *a* indicating that potato phosphorylase is different from animal phosphorylase (13). It has been long known that phosphorylase *b* requires 5′-AMP for activity while the activity of phosphorylase *a* is independent of this cofactor. However, phosphorylase I and phosphorylase II in maize endosperm are both active in the absence of cofactors. Both phosphorylases I and II are inhibited by ATP but not by glucose-6-P at the concentration tested. ATP, and glucose-6-P have no effect on phosphorylase *a* but competitively inhibit the activation of phosphorylase *b* by 5′-AMP (18).

The observations that phosphorylase II activity is not detected at some developmental stages where phosphorylase I is active, that the 2 activities are physically separable by column chromatography, and that important differences in behavior exist, suggests that these 2 phosphorylases are different enzymes. Their role in starch synthesis or degradation is not elucidated, but the appearance of the phosphorylase II activity during the period of most rapid starch synthesis and its absence during mobilization of endosperm starch during seed germination prompt speculation as to a possible synthetic role for this enzyme.

Addendum

After the preparation of this manuscript, a third type of phosphorylase activity has been detected in developing endosperms of maize. This activity is not detectable in crude homogenates nor following column chromatography since the enzyme apparently exists in an inactive form as a complex with a non-dialyzable, heat-labile inhibitor. High activity is found following protamine sulfate fractionation. Preliminary evidence indicates that the activity is not due to an altered form of phosphorylase I or phosphorylase II.

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