MUTATIONS AT THE SHRUNKEN-4 LOCUS IN MAIZE THAT PRODUCE THREE ALTERED PHOSPHORYLASES[1,2]

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WITH the recognition of enzyme systems that transfer the glucose moiety from the nucleoside diphosphate sugars to glycogen or starch, some investigators have assumed that all synthesis of starch is mediated by such enzymes, and the role of phosphorylase is purely degradative (LELOIR 1964; WHELAN 1961). On the other hand, BADENHUIZEN (1963), PORTER (1962) and HASSID (1962) have maintained that phosphorylase could not be excluded as a synthetic enzyme. Further, KRAMER, PFAHLER and WHISTLER (1958), CREECH (1965) and NELSON and RINES (1962) considering the carbohydrate storage products in maize mutants affecting starch synthesis all concluded that more than one pathway of synthesis existed: i.e., that there was more than one enzyme system active in forming α 1–4 linkages between glucose molecules. These considerations prompted an investigation of phosphorylase activity in the endosperm of developing maize seeds. The investigation revealed the existence of three different phosphorylases (I, II and III) in the endosperm (TSAI and NELSON 1968 and 1969). These phosphorylases are physically separable, appear at different times in the development of the endosperm, and respond differently to the presence of various metabolites. Phosphorylases II and III, which can initiate synthesis without a primer, are present during the period of most rapid starch synthesis but do not appear during germination. Phosphorylase I, which is present during all stages of endosperm development, reappears in the endosperm during germination.

In this paper, we present evidence that the allelic mutations sh_4^S and sh_4^A have reduced and altered enzymatic activity for all three phosphorylases. The mutation designated as $sh_4^{S(\text{tandard})}$ is the mutation first found at the locus and carried in the stocks of the Maize Genetics Cooperative at Urbana, Ill. as sh_4. The mutation designated as sh_4^A is the mutation isolated by D. W. RICHARDSON and originally named *shrunken-floury*. RICHARDSON (1956) reported that *sh-fl* was allelic to sh_4, and we have confirmed that these mutations are allelic. We are, therefore, adopting a designation indicating the allelism of *sh-fl* with a previously isolated mutation. The mutant seeds are phenotypically distinguishable with sh_4^S/sh_4^S seeds being smaller and possessing less floury endosperms than those of sh_4^A/sh_4^A seeds. It will also be shown that the two mutations result in qualitatively different phosphorylases.

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The mutant and normal endosperms have equivalent activities of invertase, hexokinase, phosphoglucomutase, adenosine diphosphate glucose (ADPG)-pyrophosphorylase, uridine diphosphate glucose (UDPG)-pyrophosphorylase, sucrose synthetase, soluble ADPG-starch glucosyl transferase, and starch-degrading enzymes.

MATERIALS AND METHODS

Self-pollinated ears of sh_4^S/sh_4^S, sh_4^A/sh_4^A, or Sh_4/Sh_4 maize (*Zea mays* L.) were harvested and quick-frozen 22 days after pollination. Phosphorylases I and II were isolated by the methods described previously (TSAI and NELSON 1968). TSAI and NELSON (1969) have described the method of preparing phosphorylase III by binding to protamine sulfate and then releasing the enzyme with a buffer solution of high ionic strength. For assays of enzyme activity, the reaction mixtures contained 10 μmoles of (2-(N-morpholino) ethanesulfonic acid) MES buffer, 0.2 μmoles of glucose-^{14}C-1-P (30,000 cpm), 5 mg of amylopectin and 10 μliters of enzyme solution containing about 3.5 μg of protein measured by the method of LOWRY *et al.* (1951) for the sh_4^S mutant phosphorylase I, 4 μg protein for the sh_4^A phosphorylase I, and 5 μg of protein for the normal maize phosphorylase I, 1 μg of protein for the sh_4^S, sh_4^A and normal maize phosphorylase II's, 2 μg for sh_4^S and sh_4^A phosphorylase III's and 1.5 μg for normal phosphorylase III. Incubation was at 37°C for varying lengths of time.

Enzymatic reactions were terminated by the addition of 0.5 ml of 0.1 N NaOH followed by 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 suspended material was distributed over a ringed planchet (3.1 cm in diameter) and evaporated under an infrared lamp before counting in a gas flow counter (Nuclear-Chicago). Each assay was made in duplicate, and the data presented are the mean values of the two assays after correction for control values from reaction mixtures that did not contain an enzyme preparation.

Electrophoretic separations were made on polyacrylamide gels containing 1% glycogen as an acceptor (a procedure suggested by Mr. T. MCDONALD) at pH 8.3 with 2.5 ma per gel. The runs were made at room temperature for 65 min. Subsequently, the gels were incubated in 0.5 M MES buffer (pH 5.8) containing 0.03 M glucose-1-P at 37°C for varying times. After the incubation period, the zones of enzymatic activity were revealed by a stain containing 0.02% iodine in 0.1% KI solution.

RESULTS

The characteristics of the three endosperm phosphorylases (TSAI and NELSON 1968 and 1969) are summarized in Table 1. Table 2 gives the specific activities of phosphorylases I, II, III, and IV isolated from self-pollinated ears of sh_4^S/sh_4^S, sh_4^A/sh_4^A, or normal maize. Phosphorylase IV is present only in the embryo. It is so designated because it is eluted from a DEAE-cellulose column by a linear NaCl gradient in the same fractions in which phosphorylase I of the endosperm would elute. It is, however, distinguishable from phosphorylase I by several criteria. The mutants clearly have much lower activity for both phosphorylase I and phosphorylase II and somewhat lower phosphorylase III activity in endosperm tissue. The activity of phosphorylase IV in the embryo is similar for all genotypes. There is no phosphorylase II activity in the embryos of either mutant or normal maize.

The sh_4^S and sh_4^A mutations were originally detected by the defective seed type they condition. The endosperms of *shrunken-4* seeds contain approximately ⅓ as much starch as normal seeds from the same ear, but the amylose percentage of

TABLE 1

*A summary of the characteristics of the endosperm phosphorylases of normal maize**

	PI	PII	PIII
First detectable in development	6 days	12–16 days†	8–12 days‡
Present during germination	Yes	No	No
pH optimum	5.8	5.9	6.5
Km (G-1-P)	3.3 mM	4.0 mM	2.0 mM
Primer requirement	Yes	No	No
Relative activity/endosperm (22 days)			
— additives	100	1000	500
+ Mg^{2+} (10 mM)	130	3500	750
+ Adenosine-5′-triphosphate (ATP) (4 mM)	55§	350¶	250§
+ Fructose-6-phosphate (F-6-P) (4 mM)	110	800	500
+ Mg^{2+}, + F-6-P	35	200	250
+ Mg^{2+}, + ATP	75	550	385
+ ATP, + F-6-P	28	880	550
+ ATP, + Mg^{2+}, + F-6-P	17	2600	750

* Adapted from TSAI and NELSON, 1969.
† Not detectable at 12 days, active at 16.
‡ Not detectable at 8 days, active at 12.
§ Inhibition by ATP is competitive.
¶ Inhibition by ATP is non-competitive.

the starch is the same (Table 3). The smaller amount of starch in the endosperms of *shrunken-4* seeds cannot be attributed to an excess of starch-degrading enzymes since the activity of such enzymes in the mutant is not higher than in normal maize.

In addition to the lower activities of the phosphorylases in $sh_4^S/sh_4^S/sh_4^S$ and $sh_4^A/sh_4^A/sh_4^A$ endosperms, the data presented in Table 4 indicate that the phosphorylases produced by the mutants are altered as compared to the enzymes produced by normal. Phosphorylase I from normal endosperms is little affected by the presence of 8.3 mM F-6-P or 8.3 mM Mg^{2+} in the reaction mixture. Both

TABLE 2

Specific activity of phosphorylases I, II, III, and IV in endosperms and embryos of sh_4^S, sh_4^A *and normal maize 22 days after pollination*

	Activity (mμmoles of glucose-^{14}C incorporated/endosperm or embryo)					
	Endosperm			Embryo		
Enzymes	sh_4^S	sh_4^A	Normal	sh_4^S	sh_4^A	Normal
Phosphorylase I	8.3	18.2	97.7	0	...	0
Phosphorylase II	21.6	27.4	983.2	0	...	0
Phosphorylase III	304.8	326.4	490.4	...	...	...
Phosphorylase IV	0	0	0	15.8	...	21.1

Reaction mixtures contained 0.2 μmole of glucose-^{14}C-1-P (30000 cpm), 10 μmoles of MES buffer, pH 5.8, 5 mg of amylopectin and 10 μl of enzyme solution in a total reaction volume of 60 μl. Incubation was at 37°C for 30 min.

TABLE 3

Starch content of mature sh_4^S, sh_4^A *and normal seeds*

Endosperms	Amount of starch (mg/endosperm)	Percent amylose
sh_4^S	66.63	26
sh_4^A	74.30	26
Normal	194.40	28

The starch was isolated according to McGUIRE and ERLANDER (1966) and the amount of amylose measured by the method of ULMANN and AUGUSTAT (1958). The sh_4^S and normal seeds were taken from an F_1 ear.

together in the reaction mixture are quite inhibitory. It is inhibited by 3.3 mM ATP, and this inhibition is reinforced by F-6-P. The two mutant phosphorylases are not inhibited more by a mixture of F-6-P and ATP than by ATP alone nor is the combination of Mg^{2+} and F-6-P markedly inhibitory.

The normal phosphorylase II preparation is much stimulated by Mg^{2+} and inhibited by ATP. The ATP inhibition is relieved by F-6-P, but the combination of Mg^{2+} and F-6-P is inhibitory. The sh_4^S preparation does not respond to the presence of magnesium; F-6-P does not relieve ATP inhibition; the combination of F-6-P plus Mg^{2+} is somewhat stimulatory. The sh_4^A preparation is much inhibited by F-6-P and little stimulated by Mg^{2+}. The presence of F-6-P somewhat relieves ATP inhibition and the combination of F-6-P and Mg^{2+} is no more inhibitory than F-6-P alone.

Other changes can be noted in the phosphorylase II produced in $sh_4^S/sh_4^S/sh_4^S$ endosperms. The pH optimum is 7.0 in contrast to the pH optimum of 5.9 in normal. The Km (G-1-P) is lower (1 mM) for the mutant enzyme than for the normal enzyme (4 mM).

TABLE 4

The effect of F-6-P, Mg^{2+}*, and ATP on the activity of the phosphorylases from* sh_4^S, sh_4^A *and normal kernels*

Additives (μmoles)			Activity (cpm)								
			Phosphorylase I			Phosphorylase II			Phosphorylase III		
F-6-P	Mg^{2+}	ATP	sh_4^S	sh_4^A	Normal	sh_4^S	sh_4^A	Normal	sh_4^S	sh_4^A	Normal
None	None	None	217	196	775	220	999	720	728	750	1550
0.5	None	None	248	218	822	256	116	602	2976	357	1562
None	0.5	None	295	445	922	269	1342	2534	5847	3007	2406
None	None	0.5	99	125	417	111	166	244	509	282	759
0.5	None	0.2	116	123	205	133	381	620	2597	1337	1685
0.5	0.5	None	193	413	244	303	90	148	2709	321	720

Reaction mixtures containing 0.2 μmole of glucose-^{14}C-1-P (30,000 cpm), 10 μl of additive, 10 μmoles MES buffer, pH 5.8, 5 mg of amylopectin and enzyme preparations as given in the text in a final volume of 60 μl were incubated at 37°C. The incubation times were 30 min for normal phosphorylase I, 150 min for sh_4^S phosphorylase I, 90 min for sh_4^A phosphorylase I; 30 min for normal phosphorylase II, and 120 min for sh_4^S and sh_4^A phosphorylase II's; 15 min for all phosphorylase III preparations.

Although the activity of the mutant phosphorylase III's is less impaired than that of the other two phosphorylases, the mutant enzymes are demonstrably altered. The sh_4^S phosphorylase III is stimulated markedly by 8.3 mM F-6-P or by 8.3 mM Mg^{2+}. The presence of both F-6-P and Mg^{2+} in the reaction mixture is not inhibitory as with the normal phosphorylase III. The Km (G-1-P) is 20 mM for the sh_4^S enzyme as compared to 2 mM for phosphorylase III from normal maize. The phosphorylase III preparation from sh_4^A differs from either the normal or sh_4^S preparations. The Km is nearly equal to that of the normal phosphorylase III—2.2 mM. Activity is inhibited by F-6-P but much stimulated by the presence of Mg^{2+}. The combination of the two is inhibitory as with normal phosphorylase III. For both mutant enzymes and the normal phosphorylase III, F-6-P relieves ATP inhibition.

The three phosphorylases prepared from normal endosperms can be separated by electrophoresis on polyacrylamide gel at pH 8.3. All three migrate towards the anode with phosphorylase I having the least mobility and phosphorylase III the greatest mobility (Figure 1). The mutant phosphorylase I's and III's cannot be distinguished from normal I or normal III on the basis of electrophoretic mobility. The sh_4^S phosphorylase II migrates less rapidly than normal phosphorylase II, and sh_4^A phosphorylase II is slower than either.

The embryos of mutant and normal seeds have approximately equal activities of phosphorylase IV (Table 2). Phosphorylase IV is not inhibited more by a

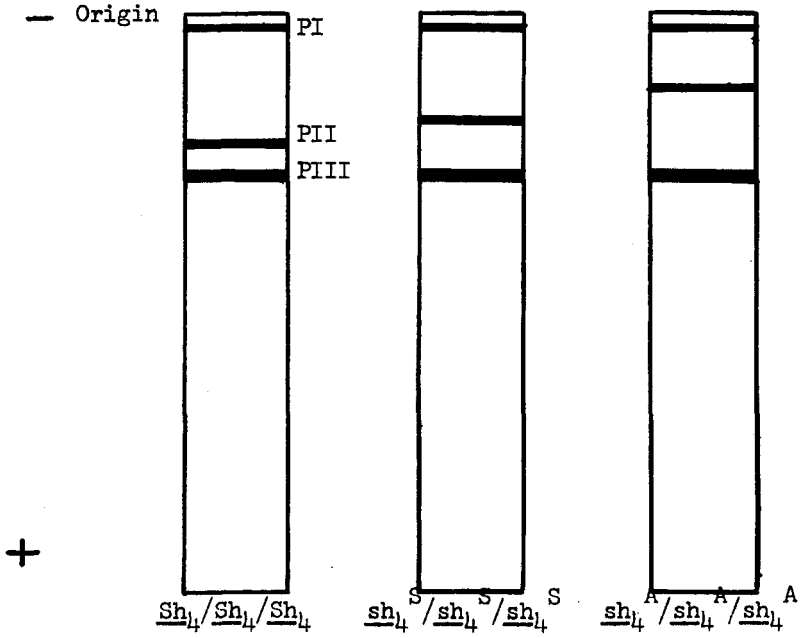

FIGURE 1.—A diagrammatic representation of the relative electrophoretic mobilities of phosphorylases I, II, and III isolated from $Sh_4/Sh_4/Sh_4$, $sh_4^S/sh_4^S/sh_4^S$, or $sh_4^A/sh_4^A/sh_4^A$ endosperms. The conditions are described under METHODS.

TABLE 5

Phosphorylase activity in endosperms and embryos of mutant and normal maize 3 days after the initiation of germination

Enzymes	Specific activity (mμmoles of glucose-^{14}C incorporated/endosperm or embryo)		
	sh_4^S	sh_4^A	Normal
Endosperm phosphorylase I	0	0	112.8
Endosperm phosphorylase II	0	0	0
Endosperm phosphorylase III	0	0	0
Embryo phosphorylase IV	1540.0	1601.6	1785.2

Reaction mixtures contained 0.2 μmole of glucose-^{14}C-1-P (30,000 cpm), 10 μmoles of MES buffer, pH 5.8, 5 mg of amylopectin and 10 μl of enzyme preparation in a total reaction volume of 60 μl. Incubation was at 37°C for 30 min.

combination of F-6-P and ATP than by ATP alone as is true of phosphorylase I. On the other hand, with phosphorylase IV, F-1,6-diP (fructose-1,6-diphosphate) relieves ATP inhibition but has no such effect on the ATP inhibition of phosphorylase I. The Km of phosphorylase I from normal endosperms is 3.3 mM while that of phosphorylase IV from normal embryos is 1.0 mM. These two enzymes are also different in electrophoretic mobility. The mobility with reference to bromphenol blue for phosphorylase I and phosphorylase IV was measured. The Rbb value for phosphorylase I is 0.05 and 0.02 for phosphorylase IV. The two enzymes can be separated electrophoretically from a mixture of phosphorylases I and IV. Additionally, the sh_4^S and sh_4^A mutations which diminish the activity of phosphorylase I markedly and alter its characteristics do not affect the activity of phosphorylase IV (Table 5). This is the second instance noted of similar but different enzymes, one of which is produced in the embryo and the other in the endosperm of maize. The same observation has been made for the starch granule-bound nucleoside diphosphate sugar to starch glucosyl transferases of the embryo and endosperm (AKATSUKA and NELSON 1966).

DISCUSSION

The altered characteristics of the mutant phosphorylases enable us to exclude the possibility that the reduced activities of the phosphorylases are secondary effects arising from reduced amount of starch synthesized. In the absence of such evidence, one might hypothesize that the production of the phosphorylases was not fully induced in the mutant or that the enzymes were not protected if produced owing to the reduced amount of starch present. The altered phosphorylases indicate a primary role of the *shrunken-4* locus in the production of the endosperm phosphorylases.

The preparations of the three phosphorylases cannot be considered to be pure in the sense of containing only the phosphorylase proteins. We have, therefore, tested the preparations for the activity of enzymes that could conceivably account for some of the interactions noted. The preparations of phosphorylase II and III

do not contain a phosphatase capable of hydrolyzing ATP, and the phosphorylase I preparations have only a very low level of ATP-ase activity. Thus the effect of adding ATP cannot be due to inorganic phosphate so liberated. We cannot detect phosphofructokinase activity in any preparation. The effects of adding both ATP and F-6-P to reaction mixtures cannot be explained by possible activity of this enzyme.

The drastic reduction in starch synthesis in the mutants which have reduced and altered activities for phosphorylases I, II and III supports the hypothesis that phosphorylase(s) have an essential role in starch synthesis. It is not clear whether the role is to provide a primer for the activity of other enzymes in the later stages of starch synthesis, to function in chain lengthening, or both. It is also possible that phosphorylase(s) act to provide a readily available source of G-1-P from a polysaccharide intermediate in starch synthesis. It should be pointed out that phosphorylases III and II which require no primer to initiate synthesis appear only after the 8th and 12th days following pollination, respectively. The ADPG-pyrophosphorylase and the soluble nucleoside diphosphate sugar-starch glucosyl transferase enzymes also appear after the 12th day just before the period of most rapid starch synthesis.

Another starch-deficient maize mutant, *shrunken-2*, which is lacking in ADPG-pyrophosphorylase activity, synthesizes only 25 to 30% as much starch as normal maize (TSAI and NELSON 1966), probably because the substrate, ADPG, for the ADPG-starch transglucosidase(s) is limiting. Like phosphorylase II, ADPG-pyrophosphorylase is not detectable 12 days after pollination but is present 16 days after pollination. It is possible that phosphorylases II and III provide primer molecules to which the ADPG-starch transglucosylases will add. It is less likely but possible that phosphorylases II and III and the ADPG-starch glucosyl transferase(s) have a coordinate role in chain lengthening such that neither operates effectively without the other. Either hypothesis would explain why the sh_2 mutation and sh_4 mutations reduce the amount of starch synthesized by approximately the same amount.

SCHWARTZ (1960) reported that a protein band present in $Sh_1/Sh_1/Sh_1$ and absent in $sh_1/sh_1/sh_1$ endosperms was much reduced in intensity in $Sh_1/Sh_1/Sh_1$; $sh_4^s/sh_4^s/sh_4^s$ endosperms. Our investigations do not reveal reduced activity for any of the three phosphorylases in $sh_1/sh_1/sh_1$ endosperms nor are there alterations in the characteristics of the three phosphorylases. It is unlikely that the band reduced in extracts of this genotype is a phosphorylase (SCHWARTZ indicated that the band had alkaline phosphatase activity). No evidence at hand indicates whether the reduction in intensity of the Sh_1 band in $sh_4/sh_4/sh_4$ endosperms is a direct or indirect effect of the sh_4 mutation.

The reduction in activity and the altered characteristics of the three phosphorylases in the sh_4/sh_4 mutant raise additional questions of interest. These enzymes make their appearance at different stages in endosperm development and have different characteristics yet are altered by the same mutational event. It is possible that the enzymes have a subunit in common, and it is this subunit that is altered by the sh_4 mutation. However, the electrophoretic mobilities of the mutant phos-

phorylase I's are not different from normal phosphorylase I nor the mobilities of the mutant phosphorylase III's from normal phosphorylase III. The mutant phosphorylase II's are both less mobile than normal phosphorylase II. These observations are not in accord with the simplest expectation from an amino acid substitution leading to a difference in net charge of a subunit that combines with three (or more) other different subunits to form the three phosphorylases. It would be expected that an amino acid substitution leading to a difference in net charge would affect all three phosphorylases unless the difference in charge were masked by the quaternary structure of phosphorylases I and III. It is also conceivable that the three enzymes are formed from the same basic polypeptide by enzymatic action or by factors influencing the tertiary structure.

SUMMARY

The recessive maize mutants sh_4^S/sh_4^S and sh_4^A/sh_4^A produce defective seeds with approximately one-third as much starch as normal seeds. Developing endosperms of the mutants (22 days after pollination) have much reduced phosphorylase I and II activity and somewhat reduced phosphorylase III activity. Phosphorylases I, II, and III from the two mutant endosperms differ from each other and from normal on biochemical criteria. The electrophoretic mobilities of the mutant phosphorylase II's are lower than normal phosphorylase II, but the mobilities of the mutant phosphorylase I's and III's are the same as their normal counterparts. The activities of other enzymes implicated in starch synthesis or degradation are equivalent in mutant and normal seeds. It is suggested that normal activity for one or more of the phosphorylases is necessary for synthesis of normal amounts of starch.

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