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A Debranching Enzyme Deficiency in Endosperms of the *Sugary-1* Mutants of Maize[1]

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ABSTRACT

Many of the *sugary-1* mutants of maize (*Zea mays* L.) have the highly branched water-soluble polysaccharide, phytoglycogen, in quantities equal to or greater than starch as an endosperm storage product in mature seeds. We find that all *sugary* mutants investigated are deficient in debranching enzyme [α-(1, 6)-glucosidase] activity in endosperm tissue 23 days postpollination and suggest that this deficiency is the primary biochemical lesion leading to phytoglycogen accumulation in *sugary* endosperms. This would indicate that the amylopectin component of starch depends on an equilibrium between the activities of branching enzymes introducing α-1,6 branch points into the linear α-1,4 glucans and debranching enzymes. The debranching enzyme activities from nonsugary endosperms can be separated into three peaks on a hydroxyapatite column. The *sugary* endosperm extracts lack one of these peaks of activity while the other two fractions have much reduced activity. The embryos of developing seeds (23 days after pollination) from both *sugary* and nonsugary genotypes have equivalent debranching activity. The debranching enzyme activity of developing endosperms is proportional to the number of copies (0 to 3) of the nonmutant (*Su*) allele present suggesting that the *Su* allele may be the structural gene for this debranching enzyme, although this is not definitive. This identification of debranching enzyme activity as being the biochemical lesion in *sugary* endosperms is consistent with several previous observations on the mutant.

The *sugary-1* (*su1*) mutant of maize, *Zea mays* L., which is the usual sweet corn of commerce, has been known and utilized since preColumbian times (21), and specific efforts to improve particular varieties of sweet corn date back to the middle of the nineteenth century (18). In spite of this lengthy association with man, the specific biochemical lesion induced by mutations at the *su* locus has not been known. Sumner and Somers (20) reported that the principal polysaccharide storage product in *su* endosperms was not starch, but a highly branched, water-soluble polysaccharide of high mol wt which Sumner and Somers referred to as phytoglycogen. Erlander (11) suggested that phytoglycogen was a normal intermediate in the process of starch synthesis and that a debranching enzyme removed some of the branches by cutting at the α-1,6 branch points. The partially debranched polysaccharide would constitute the amylopectin component of starch while the branches would be linked together to constitute the linear component (amylose). Subsequently, two laboratories (13, 15) investigated debranching enzyme activity in *su* seeds, and both reported finding such activity. It is significant, however, in view of our findings that neither laboratory simultaneously investigated debranching enzyme activity in nonsugary endosperms. In addition, an investigation of the branching patterns of amylopectin and phytoglycogen demonstrated that the branching pattern of amylopectin could not be produced by debranching phytoglycogen (16). More recently, it has been suggested (4) that *su* endosperms differ from nonsugary endosperms in possessing an anomalous branching enzyme I, but this observation has apparently not been confirmed.

We report here that *su* endosperms do have a deficiency of debranching enzyme activity relative to nonsugary endosperms. This is true of a number of *su* mutants that have arisen from independent mutational events.

MATERIALS AND METHODS

Biological Materials. Self-pollinated seeds of the maize inbred W64A or the hybrid W64A × 182E were used as the nonsugary controls. The first group of *sugary* mutants examined contained the reference allele at the *su* locus, *su-R*, and an intermediate allele *su-Bn2*. These alleles had been placed in the W64A genotype by repeated backcrosses. A second group of *sugary* alleles contained *su-8115*, *su-8116*, *su-8117*, and *su-8135*. These were presumed to have been independently induced by ethylmethanesulfonate (EMS) treatment since they were isolated in the progeny of inbred W22 plants exposed to the mutagen. For simplicity, we refer to *su* endosperms throughout the report. It should be understood that this means homozygous *sugary* (*su/su/su*) endosperms. In a similar manner, reference to nonmutant or *Su* endosperms means *Su/Su/Su* endosperms.

Developing seeds (23 d postpollination) were frozen in liquid N_2, removed from the cob, and stored at −15°C until enzyme extracts were prepared. In preparation for extraction, the pericarp and embryo were removed from each seed so that only endosperm tissue was extracted.

Phytoglycogen used as a substrate was prepared from developing sweet corn seeds (23 d postpollination) by the method of Sumner and Somers (20). Pullulan and glucoamylase were purchased from Sigma.

Enzyme Assay. Debranching enzyme activity was assayed according to the procedure of Lee *et al.* (13). The reaction mixture (150 μl for ammonium sulfate precipitates or 0.2 ml for enzyme preparations following DEAE-cellulose column fractionation) contained 1 mg pullulan, 100 mM sodium citrate buffer (pH 7.0), and 0.1 mM DTT and was incubated at 37°C. Samples were tested at intervals for an increase in reducing sugars. Glucose or maltotriose were used as standard reducing sugars for quantification of reducing sugars from reactions catalyzed by either ammonium sulfate precipitates or the enzyme from DEAE-cellulose column fractionation, respectively. Glucose or maltotriose was determined according to the method of Bernfeld (3). Phytoglycogen was extracted by a 10% TCA solution following extraction of mono- and disaccharides with a 60% methanol

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solution, precipitated with 2 volumes of 95% ethanol and estimated according to the procedure reported by Whistler and Wolfrom (23).

Activity of the glucoamylase-like enzyme was assayed in 20 mM sodium acetate buffer (pH 5.4) containing 25 mM maltose as substrate and enzyme in a final volume of 0.2 ml. The formation of glucose was measured according to the procedure recommended for STATZYM glucose (500 nm) Diagnostic Kits (Worthington Diagnostic System, Inc.).

Enzyme Preparations. Routinely, 10 g of endosperm tissue were homogenized in a mortar with 10 ml of 0.1 M citrate buffer (pH 7.0) containing 0.1 mM DTT. The crude homogenate was centrifuged at 30,000 rpm for 30 min to remove the debris. The supernatant fraction was collected for enzyme assay (preliminary testing) or precipitated with $(NH_4)_2SO_4$. The protein precipitating between 10 and 60% $(NH_4)_2SO_4$ saturation was collected and then dissolved in 10 ml of 0.1 M citrate buffer (pH 7.0) containing 0.1 mM DTT and dialyzed against the same buffer overnight before assaying. Before applying the enzyme sample to the DEAE-cellulose column, it was dialyzed again in 50 mM Tris-HCl (pH 7.0) overnight. The enzymes were eluted stepwise with increasing molarities of Tris-HCl (pH 7.0). The fractions containing debranching enzyme activity were collected, pooled, concentrated, and dialyzed against 5 mM K-phosphate (pH 7.5). The enzyme solution was applied to a hydroxyapatite column (12 × 1.0 cm), which was pre-equilibrated with the same buffer, and eluted with a linear gradient of phosphate buffer of increasing molarity. Protein was determined according to the method of Lowry *et al.* (14) using BSA as a standard or by measuring A at 280 nm.

All data concerning enzyme activity in *su* endosperms came from preparations of the reference allele *su-R* except for the data in Table I specified as pertaining to other *su* alleles.

RESULTS AND DISCUSSION

The present study demonstrates that the mutations at the *su* locus result in deficiencies of a debranching enzyme [α-(1, 6)-glucosidase]. The enzymic activities of extracts from analyses of the nonmutant and mutant genotypes as well as reciprocal intercrosses between them are presented in Figure 1. Regardless of the source of enzyme, the production of reducing sugars by hydrolysis of pullulan increased with incubation time, indicating that all genotypes tested have some debranching activity. The presence of debranching enzymes in developing maize seeds has been reported for *sugary* genotypes (13, 15). However, these studies did not test the possible difference in debranching enzyme activity between nonmutant and mutant genotypes. In this investigation, debranching enzyme activity in extracts from both normal and several *sugary* mutants was assayed simultaneously under identical conditions. A positive correlation was found between the number of copies of the *Su* allele and debranching enzyme activity. The proportionality of debranching enzyme activity with the number of *Su* alleles in the genotype suggests that the *su* locus may be a structural gene for the debranching enzymes that elute from a DEAE-cellulose column at greater than 0.2 M salt concentration. Table I gives debranching enzyme activity and the amount of phytoglycogen in a number of *su* mutants. It should be noted that *su-Bn2* is an intermediate *su* mutant in which the mature seed phenotype is much more similar to nonmutant seeds than the reference allele, *su-R* (21).

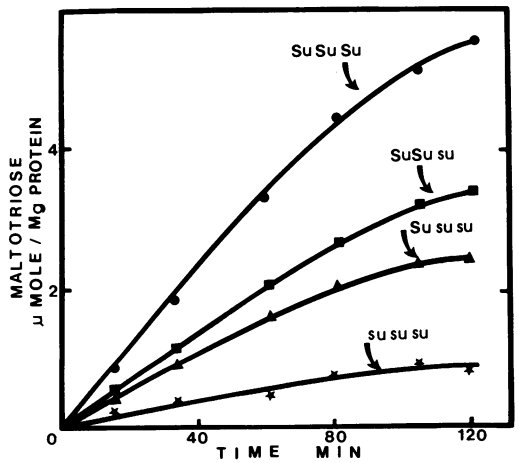

FIG. 1. Time course of digestion of pullulan by maize endosperm debranching enzyme obtained from different genotypes. Data stated as μmol of maltotriose liberated per mg protein. (●——●), Nonmutant inbred W64A *Su/Su/Su*; (■——■), *Su/Su/su*; (▲——▲), *Su/su/su*; and (★——★), *su/su/su*.

In order to analyze further the relationship between allelic state at the *su* locus and debranching enzyme activity, the enzyme preparation from nonmutant and *su-R* endosperms following $(NH_4)_2SO_4$ fractionation was further subjected to DEAE-cellulose column chromatography. The results of this procedure are shown in Figure 2, A and B. The profile of DEAE-cellulose chromatography shows that debranching activity was eluted out by 0.2 M Tris-HCl (pH 7.0). The markedly lower debranching activity in the *su-R* mutant is confirmed in these preparations. In order to rule out the possibility of the presence of either inhibitor(s) or activator(s) in enzyme preparations, which might directly modify the enzyme activity, a comparative study of the enzyme activity following sequential stages of partial purification was therefore carried out. As shown in Figure 3, A–C, debranching activity of the mutant is consistently lower at each stage of purification suggesting the lower level of mutant activity is likely due to a defect in the enzyme itself. In addition, in mixture experiments no inhibitory effect of *su* extracts on nonmutant extracts was noted (data not shown). However, if the enzyme preparation from DEAE-cellulose is further subjected to hydroxyapatite column chromatography, there is a significant difference in the elution pattern of the debranching enzyme activity (Fig. 4, A and B) in that the enzyme preparation obtained from *su* mutant shows two major fractions while the enzyme preparation obtained from nonmutant endosperm has a third enzyme fraction, which was eluted out at higher molarity of phosphate buffer. Lee *et al.* (13) noted that the debranching enzyme activity from *su* endosperms that bound to hydroxyapatite could subsequently be separated into two fractions by hydroxyapatite column chromatography. The specificities of these isoenzyme fractions were identical. Thus, the lack of a third enzyme fraction from hydroxyapatite column chromatography in the *su-R* mutant suggests that the deficiency of this particular fraction is a consequence of mutations at the *su* locus. We note, however, that there is much reduced debranching enzyme activity in the unbound fraction I eluted in hydroxyapatite column chromatography of *su* extracts.

It is interesting to note that pullulan was also hydrolyzed by the fraction eluted by 50 mM Tris-HCl from the DEAE-cellulose column (Fig. 2) indicating the possible existence of a debranching enzyme in this fraction. Since this fraction is also capable of hydrolyzing isomaltose as well as pullulan, it acts like a glucoamylase which we have found to catalyze both reactions. There is little if any R-enzyme (debranching enzyme) activity in this fraction since the apparent debranching activity is 50% inhibited in the presence of 60 mM EDTA while the debranching activity eluted later from the column is not inhibited by EDTA. Thus, a third α-(1, 6)-glucosidase in sweet corn which hydrolyzes pullulan as reported by Manners and Rowe (15) is likely a glucoamylase and not an isozyme of the major debranching enzyme.

Table II presents the K_m values with different substrates esti-

Table I. *Pullulan-Hydrolyzing Enzyme Activity in 23-Day-Old Nonmutant Su/Su/Su and Mutant (su/su/su) Endosperms*

Protein precipitated by 10 to 60% $(NH_4)_2SO_4$ saturation was used for the enzyme assay.

Genotypes	Specific Activity	Percentage of Control	Phytoglycogen Content	
	μmol/mg·min		*mg/g wet wt*	*mg/endosperm*
Su nonmutant (W64A)	0.065	100	0.18	0.0216
su-R (W64A)	0.013	21	44.50	7.40
su-Bn2 (W64A)	0.011	17	58.10	11.50
su-8135 (W22)	0.019	30	52.50	7.50
su-8115 (W22)	0.012	18	69.90	7.00
su-8116 (W22)	0.016	25	70.50	10.80
su-8117 (W22)	0.016	25	66.50	9.50

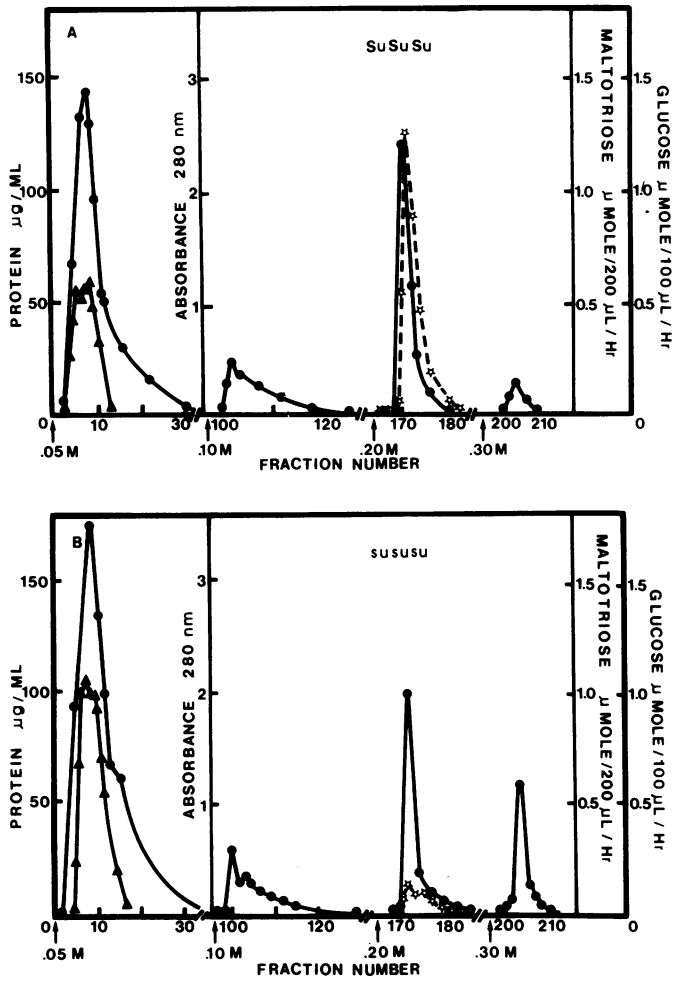

FIG. 2. DEAE-cellulose column profiles of debranching enzymes from maize endosperms of different genotypes. (●——●), Protein curve. (☆- - -☆), Debranching enzyme activity curve (μmol maltotriose liberated per 200 μl/h). (▲——▲), Glucoamylase activity curve (μmol glucose liberated per 100 μl/h). A, *Su/Su/Su*; B, *su/su/su*.

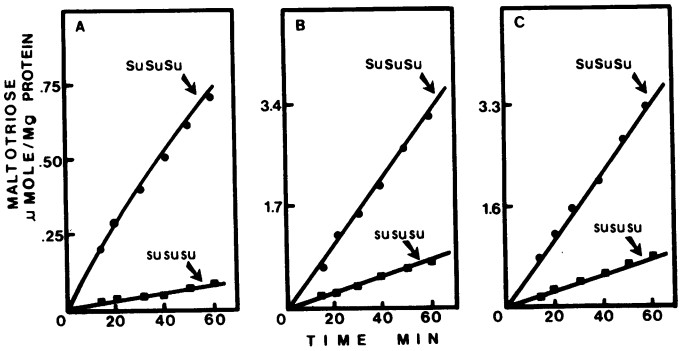

FIG. 3. Time course of digestion of pullulan by maize endosperm debranching enzyme obtained from different stages of purification. Data stated as μmol maltotriose produced per mg protein. A, Crude extract. B, After 10 to 60% $(NH_4)_2SO_4$ saturation. C, After DEAE-cellulose column chromatography.

mated for nonmutant and *su-R* enzyme preparations following hydroxyapatite column chromatography. These enzyme preparations were monitored for the presence of other enzymes (branching enzymes, disproportionating enzyme, β-amylase, and glucoamylase) that might interfere with the assay of debranching activity with negative results. Activity of these enzymes was tested under the assay conditions for debranching enzyme. The most noticeable difference, apart from the lack of the third enzyme fraction in *su* preparations, is the higher K_m for phytoglycogen for the *su* enzymes as compared to the nonsugary enzymes. This observation together with the lack of a third debranching enzyme in *su* mutant suggests to us that this missing enzyme is probably a major contributor to the development of the nonmutant phenotype. A comparative study on these three enzyme fractions is in progress.

It has been shown, in several instances in maize, that mutations affecting starch production in the endosperm are tissue-specific in that the embryos of both mutant and nonmutant seeds have equivalent activities of a different enzyme catalyzing the same reaction. Therefore, the mutation that greatly reduces or abolishes the activity of an enzyme active in the endosperm does not affect the activity of a similar enzyme in the embryo. This is true for the *waxy* locus (starch granule-bound glucosyl transferase) (1), the *shrunken-2* and *brittle-2* mutations (ADP-glucose pyrophosphorylase) (12, 18), and the *shrunken-1* locus (sucrose synthetase) (9, 10). The activity of debranching enzymes obtained from both nonmutant and *sugary* embryos of developing seeds 23 d postpollination was therefore examined. There is no significant difference in the enzyme activity of embryos from the two genotypes, suggesting that the mutation affecting debranching enzyme activity is also endosperm tissue-specific (data not shown). The ratio of debranching enzyme activity per embryo/debranching enzyme activity per endosperm for nonmutant seeds 23 d after pollination is 0.394 and for *sugary* seeds 3.318.

The pronounced deficiency of debranching enzyme in developing *sugary* endosperms as compared to nonsugary raised the question of whether during germination debranching enzyme activity in *sugary* endosperms would be comparably low. Figure 5 shows the debranching enzyme activity in the two genotypes at various times after the seeds imbibed water. It is clear that there was a marked increase in nonsugary seeds while no increase

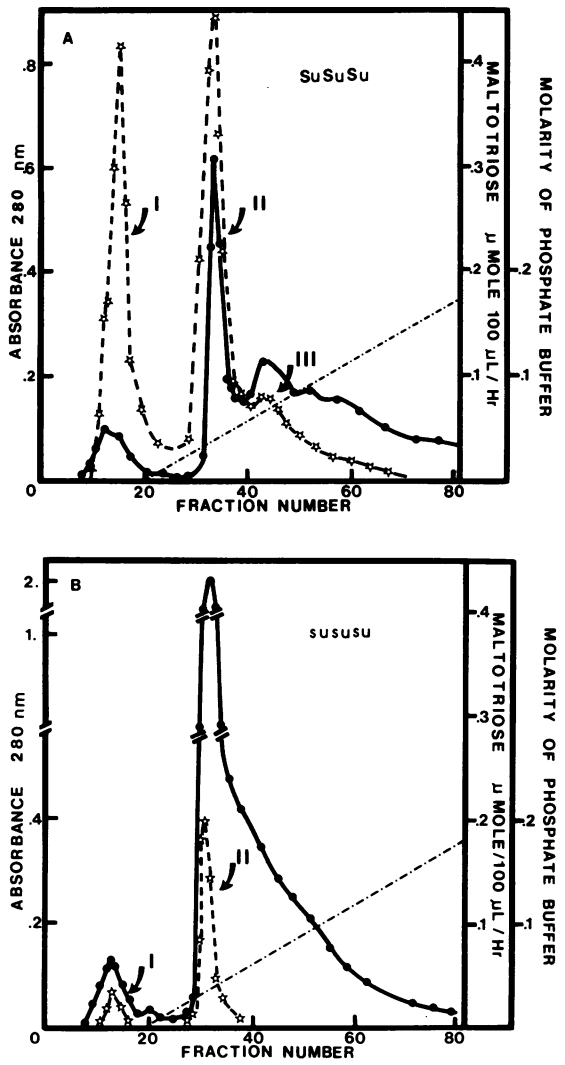

FIG. 4. Chromatography of debranching enzymes on a hydroxyapatite column equilibrated with 5 mM phosphate buffer (pH 7.5). The preparations applied to the column were the pooled fractions from DEAE-cellulose column chromatography showing debranching activity. After the initial 280 nm-absorbing peak had appeared, elution was carried out with a linear gradient of phosphate buffer frm 5 to 200 mM. (●——●), Protein curve. (☆- - -☆), Debranching enzyme activity curve (μmol maltotriose produced per 200 μl/h). A, *Su/Su/Su*; B, *su/su/su*.

Table II. *Estimates of K_m Values for Three Substrates of Debranching Enzyme Fractions from su and Nonmutant Endosperms following Hydroxyapatite Column Chromatography*

The reaction mixture contained 0.1 M sodium citrate buffer (pH 6.0), 0.1 mM DTT, polysaccharides, and enzyme (about 20–30 μg) in a final volume of 0.3 ml. The reaction mixture was incubated at 37°C for 3 h and stopped by the addition of the reducing sugar reagent (3).

Substrates	Nonmutant (*Su/Su/Su*)			Mutant (*su/su/su*)	
	I	II	III	I	II
	K_m × *mg/ml*				
Phytoglycogen	15	15	6	45.0	45.0
Amylopectin	9	4.5	9	4.5	4.5
Pullulan[a]	0.64	3.0	0.5	0.25	1.1

[a] Pullulan is not a substrate *in vivo* for maize debranching enzymes, but it is a useful substrate for determining debranching activity as pointed out by Lee *et al.* (13).

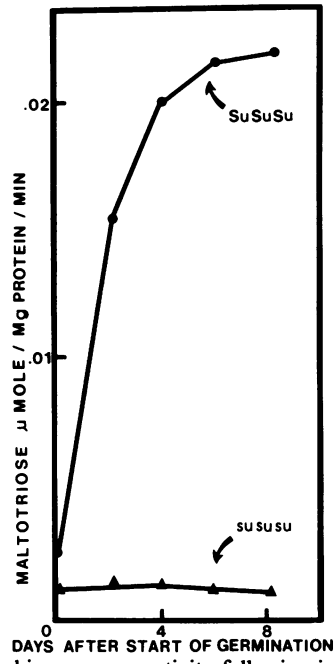

FIG. 5. Debranching enzyme activity following imbibition of water by maize seeds. Data stated as μmol maltotriose produced per mg protein per min. Nonmutant (●——●); *sugary* mutant (▲——▲).

was noted in the *sugary* seeds. This lack of increase of debranching enzyme activity with time in germinating *sugary* seeds is consistent with the observation of Manners and Rowe (15). We have no data, however, bearing on the question of *de novo* synthesis of debranching enzyme(s) as opposed to activation of a pre-existing precursor in nonmutant seeds after germination is initiated.

We can only speculate at the present time as to the reason for the apparent multiple effects of the *su-R* mutation, *i.e.* the disappearance of fraction III and the lower activities in fractions I and II. The nonmutant alleles at the *su* locus might be the structural gene for a prototypical translation product which is subjected to several different posttranslational modifications to produce debranching enzymes I, II, and III while the translation product in *su* endosperms can be processed to lower quantities of I and II and no fraction III. Another possibility is that *Su* is the structural gene for a polypeptide which as a homodimer constitutes fraction III and combines with the products of other loci to form heteromeric enzymes I and II. One could then hypothesize that the *su-R* polypeptide cannot form an active homodimer (III) but can combine with other polypeptides to make altered, less active heteromers (I and II). An examination of the debranching enzyme activity in several of the *sugary* mutants which are less defective in phenotype than *su-R* and produce less phytoglycogen may be illuminating. Our present knowledge would make it unlikely that the residual activity noted in *su* endosperms results from minor isoenzymes coded by loci other than *su*. Observations of a second, minor isoenzyme which is unaffected by a mutation abolishing the major activity have been reported in maize for the starch granule-bound nucleoside diphosphate sugar-starch glucosyl transferase (17), sucrose synthetase (9, 10), and branching enzymes (6, 7). The same situation probably applies to ADPglucose pyrophosphorylase (12). The deficiency of debranching enzyme activity in *sugary* endosperms is consistent with the accumulation in these endosperms of the highly branched polysaccharide, phytoglycogen, at the expense of starch. We believe that Erlander's hypothesis (11) that *sugary* maize is deficient in debranching enzyme activity was correct

but not his conjecture that phytoglycogen is a normal intermediate in starch synthesis. It appears rather that the amylopectin component of starch is the result of an equilibrium between branching enzyme activity and debranching enzyme activity. This equilibrium could produce the ratio of A-chains: B-chains (1.5–2.6:1) found in amylopectins by Marshall and Whelan (16) while debranching of phytoglycogen would not. When the debranching enzyme activity is deficient as in *sugary* endosperms, the resultant polysaccharide is much more highly branched.

The identification of debranching enzyme deficiency as the biochemical lesion caused by mutations at the *sugary* locus would explain also two other observations concerning the *sugary* mutants of maize. Ayers and Creech (2) have reported that *sugary*, in double mutant combinations with any other mutant affecting endosperm starch synthesis except one, produced phytoglycogen as a principal storage product. The sole exception is the *amylose extender* (*ae*) mutant which has been shown by Boyer and Preiss (6, 7) to lack the branching enzyme IIb. Thus, only the lack of a particular branching enzyme cancels the propensity of *sugary* mutants to produce phytoglycogen. Boyer *et al.* (4, 5, 8) have noted in their investigations of starch granules in *sugary* endosperms that some starch which has already been deposited within starch granules can be converted to phytoglycogen. This observation also is explicable by the deficiency of debranching enzyme activity.

LITERATURE CITED

1. AKATSUKA T, OE NELSON 1966 Starch granule-bound adenosine diphosphate glucose-starch glucosyl transferases of maize seeds. J Biol Chem 241: 2280–2285
2. AYERS JE, RG CREECH 1969 Genetic control of phytoglycogen accumulation in maize (*Zea mays* L.). Crop Sci 9: 739–741
3. BERNFELD P 1955 Amylase, α and β. Methods Enzymol 1: 149–158
4. BOYER CD, PA DAMEWOOD, EKG SIMPSON 1981 The possible relationship of starch and phytoglycogen in sweet corn. Staerke 33: 125–130
5. BOYER CD, RR DANIELS, JC SHANNON 1977 Starch granule (amyloplast) development in endosperm of several *Zea mays* L. genotypes affecting kernel polysaccharides. Am J Bot 64: 50–56
6. BOYER CD, J PREISS 1978 Multiple forms of starch branching enzyme of maize: evidence for independent genetic control. Biochem Biophys Res Commun 80: 169–175
7. BOYER CD, J PREISS 1981 Evidence for independent genetic control of the multiple forms of maize endosperm branching enzymes and starch synthases. Plant Physiol 67: 1141–1145
8. BOYER CD, EKG SIMPSON, PA DAMEWOOD 1982 The possible relationship of starch and phytoglycogen in sweet corn II. The role of branching enzyme. Starch 34: 81–85
9. CHOUREY PS 1981 Genetic control of sucrose synthetase in maize endosperm. Mol Gen Genet 184: 372–376
10. CHOUREY PS, OE NELSON 1976 The enzymatic deficiency conditioned by the *shrunken-1* mutation in maize. Biochem Genet 14: 1041–1055
11. ERLANDER S 1958 Proposed mechanism for the synthesis of starch by glycogen. Enzymologia 19: 273–283
12. HANNAH LC, DM TUSCHALL, RJ MANS 1980 Multiple forms of maize endosperm ADP-glucose pyrophosphorylase and their control by *shrunken-2* and *brittle-2*. Genetics 95: 961–970
13. LEE EYC, JJ MARSHALL, WJ WHELAN 1971 The substrate specificity of amylopectin-debranching enzymes from sweet corn. Arch Biochem Biophys 143: 315–374
14. LOWRY OH, HJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
15. MANNERS DJ, KL ROWE 1969 Studies on carbohydrate-metabolizing enzymes Part XX. Sweet corn debranching enzymes. Carbohydr Res 9: 107–121
16. MARSHALL JJ, WJ WHELAN 1974 Multiple branching in glycogen and amylopectin. Arch Biochem Biophys 161: 234–238
17. NELSON OE,PS CHOUREY, MT CHANG 1978 Nucleoside diphosphate sugar-starch glucosyl transferase activity of *wx* starch granules. Plant Physiol 62: 383–386
18. PREISS J, C LAMMEL, A SABRAW 1971 A unique adenosine diphosphoglucose pyrophosphorylase associated with maize embryo tissue. Plant Physiol 47: 104–108
19. SINGLETON WR 1944 Noyes Darling, first maize breeder. J Hered 25: 265–267
20. SUMNER JB, GF SOMERS 1944 The water-soluble polysaccharides of sweet corn. Arch Biochem 4: 7–9
21. VANDERSLICE SF, D GARWOOD 1978 Carbohydrate composition of alleles at the *sugary* locus in *Zea mays*. Agron Abstr 66
22. WELLHAUSEN EJ, LM ROBERTS, E HERNANDEZ X 1952 Races of maize in Mexico. The Bussey Institution of Harvard University.
23. WHISTLER RL, ML WOLFROM 1962 Phenol sulfuric acid colorimetric method for total carbohydrates. *In* RL Whistler, ed, Methods in Carbohydrate Chemistry, vol 1. Academic Press, New York, pp 388–389