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Characteristics of Carbohydrate Metabolism in Sweet Corn *(sugary-1)* Endosperms

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Abstract. **Metabolic characteristics of developing sugary-l maize *(Zea mays* L.) endosperms were investigated. In the later stages of development (>30 days postpollination), sugary-1 kernels maintained higher levels of many enzyme activities and retained more moisture than normal kernels. Higher enzyme activities were attributed to moisture retention and were not associated with any increase in dry weight accumulation. Of enzyme activities measured at 20 days postpollination, that of ADP-glucose pyrophosphorylase was higher in sugary-1 kernels than in normal, whereas total amylase, a-amylase, and pullulanase activities were lower. Experiments testing the effects of zero, one, two, and three doses of the sugary-1 gene in OH43 endosperms indicated that the** sugary-1 **phenotype was not expressed until three doses of the *sugary-1* gene were present. Decreased activities of amylases, but not of pullulanase, were attributed to an interference in detection by phytoglycogen. Increased ADP-glucose pyrophosphorylase activity is attributed to a response by the maize endosperm cells to increased sucrose concentrations.**

The *sugary*- 1 genotype of maize *(Zea mays),* commonly grown as sweet corn, accumulates more sugars in the endosperm than normal starchy maize. The principle difference between normal and *sugary*- 1 maize is that *sugary*- 1 endosperms accumulate the highly branched, water soluble form of starch known as phytoglycogen (Morris and Morris, 1939). There are two hypotheses concerning the origin of phytoglycogen. The first suggests that *sugary*- 1 maize kernels lack sufficient starch debranching enzyme (Erlanger, 1957). This hypothesis suggests that starch biosynthesis involves the synthesis of a highly branched intermediate form of starch which is subsequently debranched to form the amylose and amylopectin found in normal maize starch granules. Evidence supporting this hypothesis was provided by Pan and Nelson (1984), who demonstrated significantly reduced pullulanase activity (one form of starch debranching enzyme found in developing maize kernels) in *sugary*- 1 kernels that appeared to depend on the *sugary*- 1 gene dosage.

The second hypothesis suggests that the *sugary*- 1 mutation affects a starch-branching enzyme, which results in the more highly branched phytoglycogen (Boyer and Preiss, 1978). The *sugary*- 1 mutation has many additional effects on maize kernel development. Amyloplasts fail to form birefringent starch granules and instead accumulate phytoglycogen (Boyer et al., 1977). In comparison to normal kernels, *sugary*- 1 kernels accumulate less dry weight (Andrew et al., 1944; Tsai et al., 1978), retain kernel moisture longer (Andrew et al., 1944), have thinner pericarp (Andrew et al., 1944) and contain altered storage protein (Tsai et al., 1978).

In this study, we have investigated developmental and dosage effects of the *sugary*- 1 gene on carbohydrate metabolism in the maize kernel. Our results provide some additional insights into the nature of the *sugary*- 1 mutation and contribute information on the functioning of the normal maize kernel.

Materials and Methods

Plant material. Inbred and hybrid maize lines were grown in the field in Peoria, Ill., during 1986-88. Normal and *sugary*- 1 isolines of OH43 were obtained from the Maize Genetics Cooperative of the Univ. of Illinois, Agronomy Dept., Urbana. Normal and *sugary*- 1 isolines of W64A were obtained from L. Darrah, (Univ. of Missouri, Columbia). Plants grown in the field were hand-pollinated and harvested at designated time intervals. Samples were taken in a randomized design with three or four replications. For enzyme extractions and carbohydrate analyses, immature kernels were stripped from harvested ears, dissected, and about thirty endosperms were lyophilyzed and stored at -80 C until used. Whole kernels also were stripped from freshly harvested ears and frozen at -80 C until used. Kernel dry weights and moisture content were determined after drying in a forced air oven at 60C for 72 h.

Enzyme assays and carbohydrate analysis. Extraction and assays of enzymes were performed as described by Doehlert et al. (1988) and Doehlert and Kuo (1990). Enzymes were extracted by homogenizing 0.2 g powdered lyophilized tissue in 4 ml extraction buffer containing (in mM) 50 HEPES (pH 7.2), 5 $MgCl_2$, and 5 dithiothreitol. Particulates were settled by centrifugation of the homogenate at 10,000× g for 15 min.

The enzymes sucrose synthase (EC 2.4.1.13), pyrophosphate: n-fructose 6-phosphate, 1-phosphotransferase (EC 2.7.1.90; PFP), UDP-Glc pyrophosphorylase (EC 2.7.7.9), ADP-Glc pyrophos-

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Abbreviations: ADP-Glc, adenosine diphosphate glucose; DPP, days postpollination; HEPES, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid; PFK, phosphofructokinase; PFP, pyrophosphate: fructose-6-phosphate, 1-phosphotransferase; UDP-Glc, uridine diphosphate glucose.

phorylase (EC 2.7.7.27), aldolase (EC 4.1.2.13), NAD-dependent sorbitol dehydrogenase (EC 1.1.1.14), phosphoglucoisomerase (EC 5.3.1.9), phosphoglucomutase (EC 2.7.5.1), phosphofructokinase (EC 2.7.1.11), glucokinase (EC 2.7.1.11), and fructokinase (EC 2.7.1.4) were assayed by continuous spectrophotometric assays where the reaction product was coupled with either NAD reduction or NADH oxidation and monitored by A_{340} as described by Doehlert (1990) and Doehlert et al. (1988).

Pullulanase (EC 3.2.1.41) was assayed by the procedure of Doehlert and Knutson (1991). Assays contained 50 mM acetate-NaOH (pH 5.0), 5 mM $MgCl_2$, and 2.5% pullulan (w/v). Samples were taken at 0, 10, 20, and 30 min. Reducing power of the sample was determined with dinitrosalicylic acid solution (Bernfield, 1951), and compared with a maltotriose standard curve. An additional blank was used containing enzyme extract but no pullulan to correct for the contribution of hydrolyzed phytoglycogen to the increase in reducing power. Total amylase activity was assayed by measuring the increase of reducing power in a solution of soluble starch. Assays contained 50 mM citrate (pH 6.0) and 5% soluble starch (w/v). Assays were terminated after 10 min, and the reducing power was measured with dinitrosalicylic acid solution (Bernfield, 1951). The α -amylase activity was assayed by the starch azure method (Doehlert and Duke, 1983). Assays contained (in mM) 50 citrate buffer (pH 6.0), 5 dithiothreitol, 2 $CaCl_2$, and 20 g starch azure/liter. Interference by β -amylase was eliminated by adding 1000 units sweet potato β -amylase/ml. The solubilized blue pigment indicative of α -amylase activity was measured at 595 nm and was compared with that released by known activities of bacterial α -amylase in the presence of 1000 units/ml sweet potato β -amylase. Doehlert and Knutson (1991) indicated that starch debranching enzyme activities had no effect on the starch azure assay.

All enzyme assays were conducted at 30C. One unit of activity is defined as the activity necessary to produce 1 µmol of product in 1 min at 30C.

Staining of electrophoretic gels for starch hydrolytic activity was performed by electrophoretic transfer of proteins from a native polyacrylamide gel onto a starch-containing gel, followed by KI-I_2 staining using the method of Kakefuda and Duke (1984) as modified by Beers and Duke (1988). The α– and β -amylases were identified by electrophoretic transfer onto gels containing β– limit dextrin. The β -limit dextrin was prepared by the method of Swain and Dekker (1966).

To test the effects of phytoglycogen on the activities of starch hydrolytic enzymes, 10 mg of phytoglycogen/ml was added to a set of three samples of normal 20-days postpollination (DPP) whole OH43 kernel extracts, and an equal volume of water was added to a control set. All samples were then centrifuged at 30,000× g for 20 min. Subsequent to centrifugation, all samples were assayed for activities of starch hydrolytic enzymes.

Soluble sugars were extracted and identified by high pressure liquid chromatography as described by Kuo et al. (1988). Total (1:4),(1:6) α -D-glucan (starch + phytoglycogen) was determined on 50-mg samples. Samples were washed three times with 70% ethanol (v/v) to remove soluble sugars. The washed pellets were then suspended in 5 ml of 400 mM acetate buffer (pH 5.0) and heated to 90C for 90 min to gelatinize the starch. Twenty units of amyloglucosidase (Sigma #A-9268) were added to each sample along with NaF to a final concentration of 10 mM to prevent microbial growth, and samples were allowed to incubate at 40C for 48 h. Samples were centrifuged at 1200× g for 2 min, and supernatants were placed in respective 50-ml volumetric flasks. Pellets were rinsed twice and centrifuged, supernatants were pooled and brought up to 50 ml, and glucose concentrations were determined with the glucose oxidase method (Gascon and Lampen, 1968). Soluble sugars, phytoglycogen, and starch in mature *sugary* -1 gene dosage series kernels were fractionated by the procedure described by Dickinson et al. (1983) and quantitated by the phenol sulfuric acid method (Hodge and Hofrieter, 1962). Phytoglycogen was purified from OH43 *sugary*- 1 kernels by the method of Schoch (1957).

Student's *t* tests and analyses of variance were performed with the ABSTAT (Anderson Bell, Parker, Colo.) computer statistics package. Least significant differences were calculated by the method described by Steel and Torrie (1960).

Results and Discussion

During kernel development, *sugary*- 1 kernels had significantly higher fresh weight than normal kernels ($P > 0.01$; Table 1) from 10 through 35 DPP, and had significantly lower dry weight from 20 DPP to maturity ($P > 0.05$, Table 1). Likewise, *sugary*- 1 kernels had higher moisture contents at 20, 25, 30, and 35 DPP than normal ($P > 0.05$; Table 1). Normal mature (9% moisture) kernels had significantly higher fresh and dry weights than mature *sugary*- 1 kernels. Some of the differences in kernel dry weights can be attributed to losses in *sugary*- 1 kernel dry weight during the dry-down period of kernel development (after 30 DPP). The *sugary*- 1 kernels lost about 8% of their dry weight during this period, whereas normal kernels lost only 2%. A comparison of means by a Student *t* test indicated that there was a significant decrease in dry weight in the *sugary*- 1 genotype ($P > 0.01$), whereas the decrease in dry weight in the normal genotype was not significant. Similar decreases in dry weight by OH43 *sugary*- 1 kernels during the dry-down periods were also observed in trials performed in subsequent years (data not shown). A portion of the reduced dry weight in *sugary*- 1 kernels is attributed to a decrease in total (1:4),(1:6) α -D-glucan (starch + phytoglycogen) in the endosperms (Fig. 1A).

Of the enzyme activities measured in developing kernels, sucrose synthase, UDP-Glc pyrophosphorylase, aldolase, phosphoglucomutase, and phosphoglucoisomerase had increased activity in *sugary*- 1 kernels in the later stages of kernel development, especially from 25 to 35 DPP (Fig. 1). The enhanced enzyme activities at these stages of kernel development in *sugary*- 1 kernels may be attributable to the retention of moisture. Cox and Dickinson (1973) may have been observing a similar phenomenon when they described increased hexose kinase activities in *shrunken-2* kernels at the later stages of development. The retention of moisture also

Table 1. Fresh weight, dry weight, and percent moisture of normal and *sugary*-1 OH43 kernels at various days postpollination (DPP).[z]

	Fresh wt (mg/kernel)		Dry wt (mg/kernel)		Moisture (%)	
DPP	Normal	*sugary*-1	Normal	*sugary*-1	Normal	*sugary*-1
10	80	112**	15	16	82	85
15	139	192**	38	35	73	82
20	255	312**	108**	91	58	71**
25	295	386**	156**	142	53	63*
30	342	466**	192*	182	44	61**
35	315	426**	207**	178	34	58*
Mature kernel	223	186**	202**	169	9	9

[z]Mean of 30 individual kernels.
*,**Significant difference between normal and *sugary*-1 at $P = 0.05$ and 0.01, respectively.

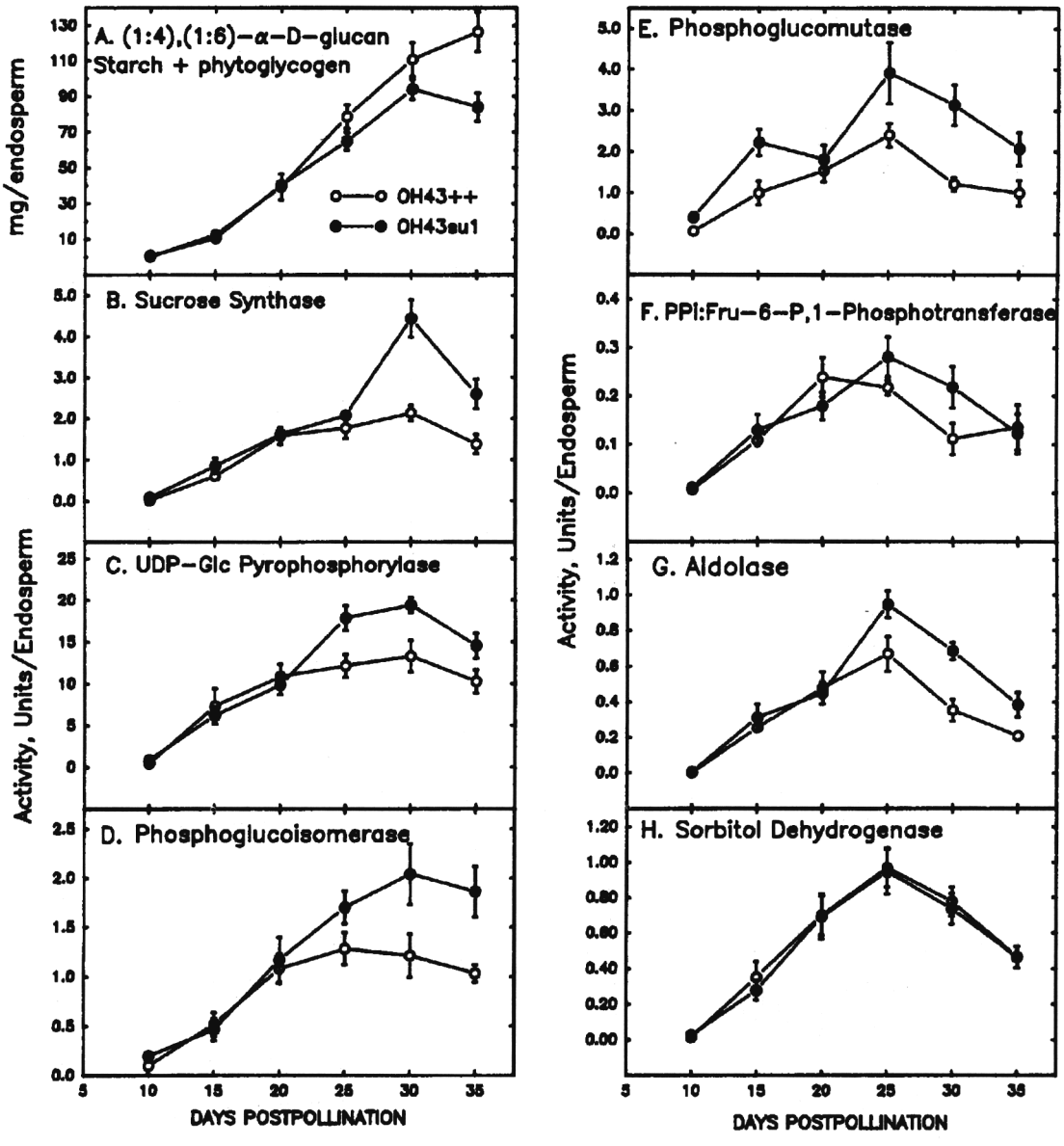

Fig. 1 . Profiles of enzyme activities and (1:4),(1:6) α-D-glucan (starch + phytoglycogen) accumulation in endosperms from normal and *sugary*-1 OH43 maize during maturation. One unit of activity is the activity required to produce 1 µmol of product in 1 min. Error bars indicate SE.

Table 2. Sugar concentrations (mg/g dry wt) in normal and *sugary*-1 OH43 maize endosperm at various days postpollination (DPP).[z]

	Sucrose		Glucose		Fructose		Sorbitol	
DPP	Normal	*sugary*-1	Normal	*sugary*-1	Normal	*sugary*-1	Normal	*sugary*-1
10	229	205	117	104	86	82	6.6	10.6**
15	51	121**	4	53**	3	33**	0	8.4**
20	41	124**	3	10	3	5	0	5.3**
25	41	94**	1	15*	2	3	0	3.3**
30	19	43**	0	14*	0	2	0	3.3**
35	19	70**	0	33**	0	6	0	5.0**

[z]Mean of three extractions, each from kernels frcm a separate ear.
*,**Significant difference between normal and *sugary*-1 at $P = 0.05$ and 0.01, respectively.

may have enhanced respiratory rates in *sugary*- 1 kernels, which may have contributed to the dry weight losses observed during the dry-down period (Table 1).

An analysis of endosperm sugars during development indicated that *sugary*- 1 kernels in general had higher sugar concentrations than normal kernels (Table 2). Sucrose and glucose were higher in *sugary*- 1 kernels than in normal from 15 through 35 DPP, and sorbitol was higher at all dates sampled. Sorbitol is produced by sorbitol dehydrogenase (Doehlert, 1987), whose activity was not significantly different in normal and *sugary*- 1 endosperm (Fig.

Table 3. Enzyme activities in crude endosperm extracts from normal (+++) and *sugary*- 1 *(su*- 1) isolines of OH43 and W64A backgrounds, starchy (A619 x W64A), and sugary- 1 ('Golden' x 'Bantam') maize hybrids.[z]

Genotype	Gluco-kinase	Fructo-kinase	PFP[y]	Sucrose synthase	Total amylase	α-amylase	Pullu-lanase
			[μmol product/(min g dry wt)]				
OH43							
+++	0.677	0.530	2.96	23.72	5.77	1.18	0.814
su-1	0.570	0.570	2.70	22.60	2.56	0.24	0.212
W64A							
+++	0.507	0.483	3.13	31.70	8.00	2.03	0.827
su-1	0.423	0.380	4.60	28.00	4.00	0.30	0.300
A619 x W64A	0.520	0.427	5.40	20.30	4.90	2.33	0.814
Golden x Bantam	0.330	0.250	9.49	24.40	1.32	0.54	0.212

[z]Mean of three extractions.
[y]PFP = pyrophosphate: fructose 6-phosphate, l-phosphotransferase.

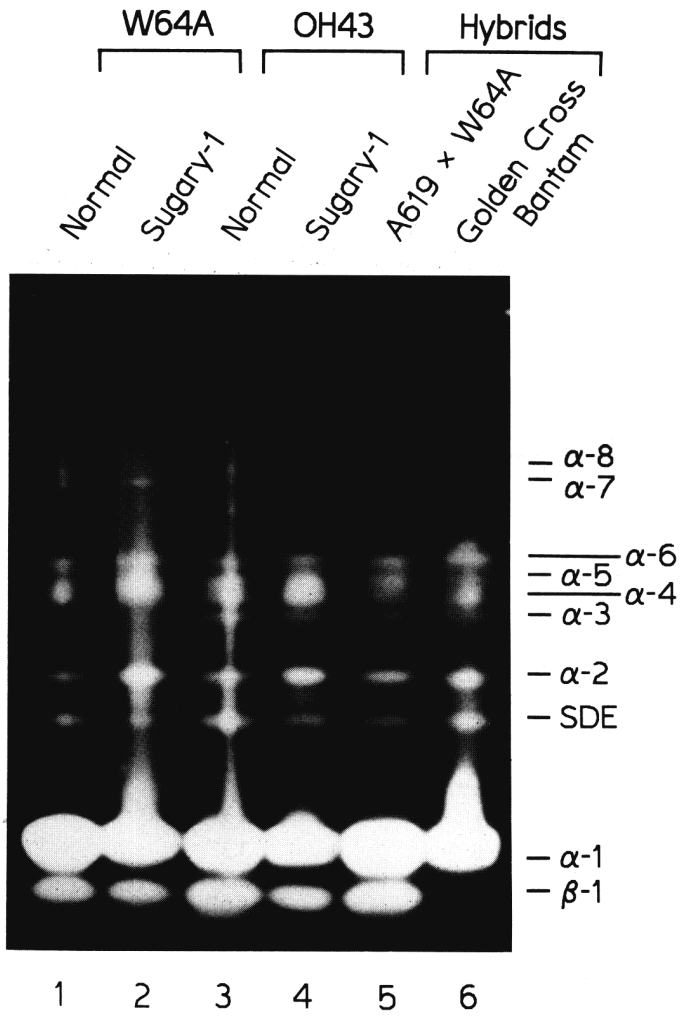

Fig. 2. Starch hydrolytic zymograms from normal and sugary-l isolines of W64A and OH43 as well as a sugary-l hybrid ('Golden' x 'Bantam') and a normal hybrid (A619 x W64A). β– 1 indicates a β– amylase; α– 1, -2, -3, -4, -5, -6, -7, and -8 are α– amylase isozymes; and SDE (starch debranching enzyme) designates the pullulanase activity band.

1H). Increased sorbitol may be a consequence of the overall increase in sugars. Fructose concentration was significantly higher in *sugary*- 1 than in normal endosperms only at 15 DPP.

Enzyme activities were measured in 20 DPP kernels of OH43 and W64A normal and *sugary*- 1 isolines as well as in the sugary-1 hybrid 'Golden' x 'Bantam' and the starchy hybrid A619 x W64A. Data (Fig. 1) indicate that enzyme activities measured at 20 DPP are not confounded by moisture retention effects. There were no consistent differences between *sugary*- 1 and normal genotypes in activities of glucokinase, fructokinase, PFP, or sucrose synthase (Table 3). However, *sugary*- 1 genotypes had consistently lower activities of total amylase, α– amylase, and pullulanase (Table 3). The lower pullulanase activity observed is consistent with results reported by Pan and Nelson (1984). Zymograms of starch hydrolytic activities from normal and *sugary*- 1 genotypes indicated no qualitative differences in isozymes of starch hydrolytic enzymes (Fig. 2), although the staining intensity of β– amylase from 'Golden' x 'Bantam' was considerably lower than those of other genotypes. We cannot attribute the observed differences in enzyme activities to the absence of any particular form of enzyme.

We produced reciprocal crosses between sugary-1 and normal lines of OH43 to produce developing kernels with zero, one, two, and three doses of the *sugary*- 1 gene. No significant differences in carbohydrate composition were observed until three doses of *sugary*- 1 gene were present in the developing endosperm. Endosperm containing three doses of the sugary-1 gene had higher soluble sugars and phytoglycogen, decreased starch, and no difference in total carbohydrate concentrations than the others (Table 4). Ayers and Creech (1969) also have shown that three doses of the *sugary*- 1 gene are necessary for significant accumulation of phytoglycogen.

Differences in enzyme activities in 20 DPP endosperms were

Table 4. Effect of *sugary*- 1 gene dosage on mature OH43 kernel carbohydrate composition.[z]

Genotype[y]	Soluble sugars	Phyto-glycogen	Starch	Total carbohydrate
		(mg/g dry wt)		
+ + +	44.1	9.8	658.8	712
+ + su	50.2	13.7	641.5	668
su su +	45.6	12.8	644.2	702
su su su	87.4	452.6	192.5	732

[z]Mean of four extractions, each from kernels from separate ears.
[y]Zero, one, two, and three doses, respectively, of the *sugary*- 1 gene (su).

Table 5. Dosage effects of the *sugary*-1 (su) gene on enzyme activities in extracts of endosperms from OH43 kernels harvested 20 days postpollination (DPP).[z]

Genotype[y]	ADP-Glc pyrophosphorylase	PFK	PFP	Fructo kinase	Gluco-kinase	α-amylase	Total amylase	Pullu-lanase
				(μmol product/min g dry weight)				
+ + +	1.00	0.793	10.3	0.116	0.308	1.43	2.72	1.86
+ + su	1.78	0.716	9.4	0.211	0.348	1.32	2.39	1.85
su su +	1.36	0.780	11.1	0.196	0.358	1.34	1.81	1.61
su su su	3.30	0.952	11.4	0.290	0.296	0.84	1.06	1.01

[z]Mean of four extractions. See abbreviation list at the bottom of the first page.
[y]Zero, one, two, and three doses, respectively.

not apparent until three doses of the *sugary*- 1 gene were present (Table 5). In accordance with data shown in Table 3, total amylase, a-amylase, and pullulanase activities were lower in *sugary*- 1 kernels than in kernels with either zero, one, or two doses of the *sugary*- 1 gene, and activities of glucokinase, fructokinase, PFP, and PFK were not affected by the *sugary*- 1 locus. In addition, ADP-Glc pyrophosphorylase activity increased in endosperms containing three doses of the *sugary*- 1 gene. Increased ADP-Glc pyrophosphorylase activity also has been observed in endosperms of starch-deficient maize mutants (Doehlert and Kuo, 1990) and in endosperms of high-starch maize lines (Doehlert and Lambert, 1991) and was attributed to increased sucrose levels in these tissues. It is consistent with these studies that the increased sucrose concentration in *sugary*- 1 endosperm would result in increased ADP-Glc pyrophosphorylase activity. Activities of PFP, glucokinase, fructokinase, total amylase, and pullulanase (Table 5) appear to differ significantly from activities of the same enzymes measured in OH43 endosperms (Table 3). Data from these tables were obtained from corn grown in different years, and we attribute the differences to developmental differences brought about by environmental conditions in these years.

The lack of correlation between the decreased activities of starch hydrolytic enzymes and *sugary*- 1 gene dosage is inconsistent with the hypothesis that the *sugary*- 1 mutation encodes the structural genes for any of these enzymes. This observation conflicts with work by Pan and Nelson (1984), who found a close correlation between *sugary*- 1 gene dosage and pullulanase activity. We have no explanation for this discrepancy, except for the fact that Pan and Nelson (1984) assayed their extracts as ammonium sulfate precipitants at pH 7.0, instead of using crude extracts at pH 5.0, the optimal pH for this enzyme (Doehlert and Knutson, 1991). The *brittle*- 1 mutant also has been reported to contain very low pullulanase activity (Doehlert and Kuo 1990). Since the work of Pan and Nelson (1984), an additional form of starch debranching enzyme of the isoamylase class has been described in developing maize kernels (Doehlert and Knutson, 1991). Interference by phytoglycogen and endogenous amylases has prevented us from testing for isoamylase activity in *sugary*- 1 endosperms.

A major problem in assaying starch hydrolytic enzymes in *sugary*- 1 endosperm extracts lies in the probable interference by the water soluble phytoglycogen. We found that aqueous *sugary*- 1 extracts are milky from solubilized phytoglycogen in comparison with normal extracts, and contained up to 4 mg phytoglycogen/ml (data not shown). This amount represents a significant endogenous substrate pool for starch hydrolytic enzymes and poses other more subtle problems that aggravate attempts to quantitate enzyme' activities. In an experiment to determine the effects of phytoglycogen on starch hydrolytic enzyme activities, we added purified phytoglycogen to normal whole kernel extracts and centrifuged the preparations. We then assayed starch hydrolytic enzymes in the supernatants. We found that this treatment significantly decreased both total and α -amylase activitites in the extracts. The mean activity of pullulanase was lower in the phytoglycogen-treated samples; the difference was not significant at $P \leq 0.05$ but was significant at $P \leq 0.1$ (Table 6). Presumably, the amylases bind to the phytoglycogen as they would in a glycogen precipitation commonly used to purify α -amylase (Thoma et al,, 1971). Centrifugation pellets a portion of the phytoglycogen and with it, a portion of the amylases. Pullulanase may bind to phytoglycogen to a lesser extent, but these results do not account for all of the decreased pullulanase activity found in *sugary*- 1 extracts. We also have tested the effects of phytoglycogen concentration on pullulanase and amylase activities, and found no effect of phytoglycogen, up to 10 g·liter^{-1}, on either pullulanase or α–amylase activities, as long as the contribution of phytoglycogen hydrolysis was subtracted from the total hydrolytic activity (data not shown). Thus, we cannot account for all of the decreased pullulanase activity from phytoglycogen interference. It seems likely that the *sugary*- 1 mutation reduces expression of pullulanase. However, evidence suggests that pullulanase is a monomer, and the multiple forms of pullulanase observed by Pan and Nelson (1984) and others (Toguri, 1991) are different configurations of the same peptide (Beck and Zeigler, 1989).

In conclusion, we have presented several metabolic characteristics that are the consequence of the *sugary*- 1 mutation, including increased activities of specific enzymes during kernel dry-down,

Table 6. Effect of phytoglycogen on the recovery of extracted starch hydrolytic enzyme activity in corn kernel extracts following centrifugation. All samples were derived from the same normal OH43 whole kernel extract.[z]

Enzyme	Control	Control + phytoglycogen	*t*	*P*
	(μmol product/ml extract ± SD)			
Total amylase	0.853 ± 0.081	0.739 ± 0.062	–9.913	0.0050
α-amylase	0.056 ± 0.021	0.014 ± 0.001	–3.561	0.0353
Pullulanase	0.051 ± 0.002	0. 044 ± 0.006	–2.452	0.0669

[z]Mean from three experiments.

increased ADP-Glc pyrophosphorylase activity at 20 DPP, and decreased starch hydrolytic enzyme activities at 20 DPP. Because these events are correlated with the *sugary*- 1 phenotype and not the sugary-l gene dosage, it appears that these events are a consequence of the sugary-1 lesion, rather than the primary effect.

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