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# Carbohydrate and Enzymic Characterization of a High Sucrose Sugary Inbred Line of Sweet Corn[1]

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## ABSTRACT

**Reserve carbohydrates were determined on developing endosperm of a new line of sugary maize (*Zea mays* L.). Other entries, included for comparative purposes, were Midway (sugary), Funks G4646 (starchy), and Illini X-tra Sweet (shrunken-2). Sucrose in the new line, Illinois 677a, was more than twice that of Midway at most stages of development, and reached a maximum of 40% of dry weight at 18 days after pollination. Appreciable phytoglycogen accumulated in Illinois 677a, reaching 30% or more of dry weight as endosperm tissue matured. Thus, Illinois 677a is a typical sugary maize concerning phytoglycogen content, but it resembles shrunken-2 concerning the extent of sucrose accumulation.**

**Enhanced sucrose accumulation by Illinois 677a was not accounted for by altered *in vitro* activities of invertase, sucrose synthase, UDP-glucose pyrophosphorylase, or ADP-glucose pyrophosphorylase. Its normal level of ADP-glucose pyrophosphorylase set Illinois 677a apart from shrunken-2 in which the enzyme was drastically reduced.**

Many maize mutants are known that alter the composition of endosperm carbohydrates (4, 21). The sugary *(su)* genotype, commonly used as sweet corn, has more sucrose than starchy maize. Sugary maize is unique in accumulating phytoglycogen, a water-soluble $\alpha$-1,4-glucan that is more highly branched than the amylopectin components of maize starch (22). Several maize mutants in the shrunken *(sh)* and brittle *(bt)* series are reported to have as much as twice the sucrose content of sugary *(su)*, but phytoglycogen does not accumulate (2, 3, 11, 16).

Maize plants that are homozygous for two or three carbohydrate-modifying genes have been bred in attempts to obtain more sucrose than sugary while retaining phytoglycogen (2, 3). Several of these mutants possessed increased endosperm sugars, but in each case, the phytoglycogen was reduced (2, 3). Phytoglycogen was not reduced in endosperms that had various combinations of the genes sugary-2 *(su2)*, dull *(du)*, and waxey *(wx)* in addition to *su*, but unfortunately, the sugar content was not consistently increased in any of these compared to *su* alone (3).

Hexoses are the starting point for synthesis of sucrose and $\alpha$-glucans by developing maize endosperm; translocated sucrose is apparently hydrolyzed prior to its arrival in the endosperm cells (25). It is not clear whether an inability to obtain high sucrose and phytoglycogen in the same tissue is due to competition for common precursors or to some other reason. Interpretation is made difficult by uncertainties as to whether ADP-glucose is the sole substrate for biosynthesis of all maize $\alpha$-glucans (21), and whether phytoglycogen arises by increased branching of amylopectin or by a separate pathway (10, 18, 23).

We recently reported that a new inbred line, Illinois 677a, possessed a total sugar content equal to that of *sh2* and it remained tender and edible for a longer period than other *su* lines (8). Since kernels of Illinois 677a possessed the creamy consistency of *su* rather than the watery consistency of *sh2*, it seems likely that phytoglycogen was present. Hence, a carbohydrate analysis of Illinois 677a endosperm was carried out and the results are presented below. Selected enzymes were also assayed in hopes of learning the mechanism of sugar accumulation. Comparisons were made among Illinois 677a, a standard *su* sweet corn hybrid, an *sh2* hybrid known to have high sucrose, and normal or starchy maize. Two *su* inbreds, parents of Illinois 677a, were included in certain of the comparisons.

## MATERIALS AND METHODS

### PLANT MATERIALS

The plant materials used in this study were the following mutant and normal genotypes of *Zea mays* L.: Illinois 677a, an *su (su-677a)* $F_9$ generation inbred; Midway, an *su* hybrid; Illini X-tra Sweet, an *sh2* hybrid; and Funks G4646, a starchy hybrid. Illinois 677a is an inbred line developed at the University of Illinois and comes from a three-way cross: (Bolivia 1035 × Illinois 44b) × Illinois 442a (8). Bolivia 1035 is an interlocking Coroico flour corn; Illinois 44b and Illinois 442a are *su* inbreds of high quality, and both were included in certain of the enzymic studies. All plant materials were grown at the Vegetable Crops campus plots of the University of Illinois at Urbana-Champaign in the summer of 1973. Materials were planted May 30 at row spacing of 90 cm and 30 cm within rows. Controlled pollinations were carried out to ensure correct kernel age, uniformity of maturation, and to prevent cross-pollination. At predetermined stages of development, at least three ears, unless otherwise stated in the results, were randomly harvested from the genotypes under study. The husks and silk were rapidly removed and the ears immediately frozen in liquid nitrogen. Each frozen ear was placed in a plastic bag, labeled, and stored at −15 C. The times from harvest to freezing and from harvest to storage were generally within 15 and 30 min, respectively. In all cases, kernel age refers to the number of days between pollination and harvest.

### CHEMICALS

Most biochemicals were purchased from Sigma Chemical Co., except dithiothreitol and MES buffer, which were from Calbiochem. Crystalline BSA was from Miles Laboratories. DMSO[3]

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[3] Abbreviation: DMSO; dimethylsulfoxide.

was from Fisher Scientific, and sodium pyrophosphate-$^{32}$P was from New England Nuclear. All chemicals to be used for enzyme extraction or assay were dissolved in glass-distilled $H_2O$.

### EXTRACTION AND DETERMINATION OF CARBOHYDRATES

Carbohydrates were extracted according to Shannon (24) with some modifications. For each genotype, one sample/ear and at least three ears/stage of development were extracted. Optimum extraction procedures were established using Midway kernels at five different stages of development (7), and a standard procedure was adopted as described below. Ten g of frozen kernels taken from the center portion of each ear were ground with 50 ml of boiling 80% (v/v) ethanol for 1 min in a Sorvall Omni-Mixer (16,000 rpm under normal load). For 45-day kernels, a boiling time of 5 min with the 80% ethanol was necessary to soften the kernels before grinding. The homogenate was centrifuged at 13,000*g* for 6 min at 20 C. The pellet was resuspended with 50 ml of boiling 80% ethanol and centrifuged again. The process was repeated a third time, and the three supernatants were combined and taken to a final volume of 250 ml (supernatant I). The pellet was resuspended in 50 ml of 10% (v/v) ethanol and stored overnight at 4 C to allow phytoglycogen to dissolve. Then, the homogenate was centrifuged at 13,000*g* for 6 min at 20 C, the supernatant was decanted, and the pellet was resuspended and centrifuged again in the same manner three additional times. The four resulting supernatant fluids were combined and taken to a final volume of 250 ml (supernatant II). The remaining pellet was resuspended in 50 ml of 95% (v/v) DMSO and incubated for 2 hr at 70 C ± 3 C with constant stirring. The homogenate was then equilibrated to room temperature and centrifuged at 13,000*g* for 6 min at 20 C. The pellet was extracted with a second 50-ml portion of 95% DMSO as described above, and the supernatant fluids were combined (supernatant III). For kernel ages of 30 days or more, 75 ml of DMSO instead of 50 ml were used in each extraction. The supernatant fluids were stored at −18 C until use.

Carbohydrates in the various extracts were determined spectrophotometrically with D-glucose as the standard. Triplicate determinations were done on each extract. Total sugar determinations were done on supernatant I using the anthrone procedure of Hassid and Neufeld (9). Reducing sugars were determined with the arsenomolybdate method of Nelson (20). Sucrose was determined by subtracting reducing sugars from total sugars. Since sucrose, glucose, and fructose were the only sugars seen on thin layer chromatograms of extracts from Illinois 677a kernels harvested at 18 and 30 days, the indirect determination of sucrose was considered valid.

The phytoglycogen content of supernatant II and starch content of supernatant III were determined with anthrone reagent as described above for total sugars.

### DRY WEIGHTS

Dry weights were determined by drying samples at 68 C for 48 hr in a forced air oven.

### PREPARATION OF KERNELS FOR ENZYME ASSAYS

Enzyme assays were done on 22-day material. From 20 to 30 frozen kernels from the middle portion of an ear were cut above the pedicel and freed of pericarp and embryo tissues. To keep the tissue chilled, the working surface during dissection was a glass Petri dish filled with ice. The endosperms were weighed and ground with 2 volumes (v/w) of extraction buffer for 2 min with a VirTis "45" homogenizer with the glass flask of 2- to 30-ml capacity immersed in an ice water bath. The extraction buffer contained 50 mM K-phosphate (pH 7), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM KCl. Grinding was carried out at a power setting of 60 and at 15-sec intervals to prevent heating. The homogenates were centrifuged at 25,000*g* for 20 min at 1 C, and the resulting supernatant fluid was the source of enzyme.

### PROTEIN DETERMINATION

Protein was precipitated from 50-$\mu$l aliquots of enzyme extracts with 0.5 ml of ice cold 5% (v/v) trichloroacetic acid, resuspended in 1 ml of 0.1 M NaOH containing 2% (w/v) $NaCO_3$, and determined by the method of Lowry *et al.* (17). Crystalline BSA was the standard.

### ENZYME ASSAYS

All enzymes were assayed at 30 C under conditions such that enzyme activity was proportional to amount of enzyme used. Duplicate assays were done on each extract.

**Invertase.** Invertase ($\beta$-fructofuranosidase, EC 3.2.1.26) was assayed at pH 4.8 by measuring the reducing sugars released after incubation with sucrose (28). The supernatant fluid was dialyzed 16 to 18 hr before assay for soluble invertase. The particulate fraction was washed three times with extraction buffer and then assayed for bound invertase. Two different extractions were done for each entry, except that there was one extraction each for Illinois 44b and for Illinois 442a, so results from the latter two were not included in the statistical analysis.

**Sucrose Synthase.** Sucrose synthase (UDP-glucose:D-fructose 2-$\alpha$-glucosyltransferase, EC 2.4.1.13) was determined at pH 7.5 in the direction of sucrose formation according to Murata (19). Sucrose synthase in the cleavage reaction was assayed at pH 6 according to Murata (19), except that UDP-glucose was measured spectrophotometrically by coupling to the UDP-glucose dehydrogenase reaction (7). There were either four or five extractions for each entry.

**Sucrose Phosphate Synthase.** Sucrose phosphate synthase (UDP-glucose:D-fructose-6-phosphate 2-$\alpha$-glucosyltransferase, EC 2.4.1.14) was assayed as indicated above for sucrose synthase in the direction of sucrose formation, but fructose 6-P was used instead of fructose.

**ADP-Glucose Pyrophosphorylase.** ADP-glucose pyrophosphorylase (ATP: $\alpha$-D-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) was determined by measuring the formation of [$^{32}$P]ATP from ADP-glucose and sodium pyrophosphate-$^{32}$P (6). Six different enzyme extracts were made of Illinois 677a and each of the three hybrids. Two extracts were made on Illinois 442a and Illinois 44b, and these results were not included in the statistical analysis.

**UDP-Glucose Pyrophosphorylase.** UDP-glucose pyrophosphorylase (UTP: $\alpha$-D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9) was determined by the procedure described for ADP-glucose pyrophosphorylase, modified somewhat (7) to improve retention of labeled UTP. There were two to seven enzyme extracts/entry, with four extracts made for Illinois 677a, five for Midway, four for Funks G4646, and seven for Illini X-tra Sweet.

### STATISTICAL ANALYSIS

For carbohydrates, a split plot experiment with the main plots arranged in a completely randomized design was used to analyze the results. Genotypes were main plots, and kernel ages were split plots. There were three replicates (three ears/genotype·kernel age), and three determinations/replicate. For the enzyme studies, completely randomized designs were used with genotypes as treatments. The number of replicates varied with the enzyme and the genotype considered. Least significant difference values calculated at the 5% level of probability were used to compare treatment means (26).

## RESULTS AND INTERPRETATION

### CARBOHYDRATES

Results of the sugar analyses are presented in Figures 1 to 3. The total sugar content of Illinois 677a was high, generally resembling that of *sh2* and being more than twice that of Midway and Funks G4646 at all but the earliest developmental stage (Fig. 1). These results agree well with the earlier comparison of Illinois 677a with three standard *su* hybrids and with *sh2* (8). The reducing sugar content of all four maize entries was about the same except at 14 days (Fig. 2). Hence, enhanced sucrose accumulation was responsible for the increased total sugar content of Illinois 677a from 18 days onward (Fig. 3).

Results of the polysaccharide analyses are presented in Figures 4 and 5. The phytoglycogen content of Midway ranged from about 25 to 40% of dry weight at various developmental stages (Fig. 4), levels that are typical for an *su* genotype (3). The phytoglycogen content of Illinois 677a closely resembled that of Midway at all but the earliest stage. In agreement with reports by others (3, 16), there were only traces of carbohydrate in the phytoglycogen fractions from the *sh2* and starchy genotypes.

Illinois 677a differs from the other three entries in its significantly lower starch content (Fig. 5). It was expected that the three sweet corn hybrids would contain less starch than Funks G4646, but it was not expected that Illinois 677a would contain less starch than Midway and Illini X-tra Sweet. In percentage of total carbohydrate, there is no marked developmental trend or persisting difference among Illinois 677a, Midway, and Funks G4646 (Fig. 6). The percentage of total carbohydrate of the *sh2* hybrid tends to decrease with time and is significantly less than the other entries at 45 days after pollination (Fig. 6). These observations are in keeping with the collapsed appearance of mature *sh2* kernels, whereas Illinois 677a has the wrinkled kernel characteristic of an *su* genotype.

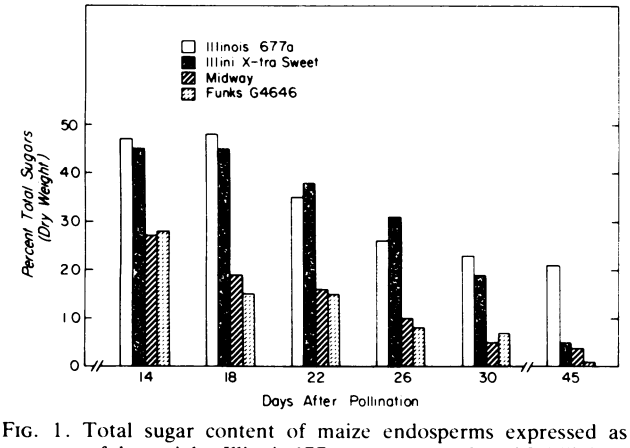


FIG. 1. Total sugar content of maize endosperms expressed as a percentage of dry weight. Illinois 677a was compared to *sh2* (illini X-tra Sweet), *su* (Midway), and normal (Funks G4646) hybrids at different stages of kernel development. For genotypes among days after pollination, the $LSD_{(0.05)}$ = 9.02%. For genotypes within days after pollination, the $LSD_{(0.05)}$ = 8.96%.

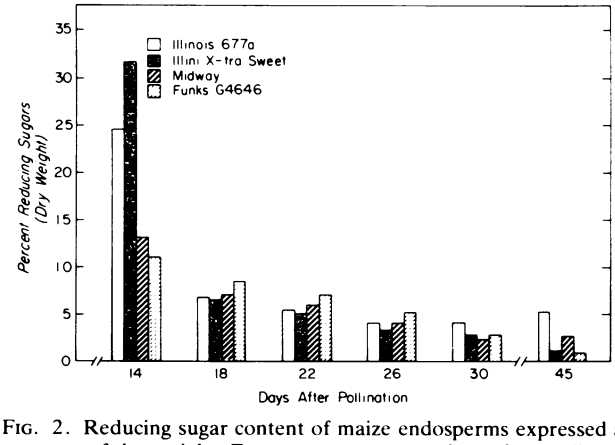


FIG. 2. Reducing sugar content of maize endosperms expressed as a percentage of dry weight. For genotypes among days after pollination, the $LSD_{(0.05)}$ = 5.58%. For genotypes within days after pollination, the $LSD_{(0.05)}$ = 5.52%.

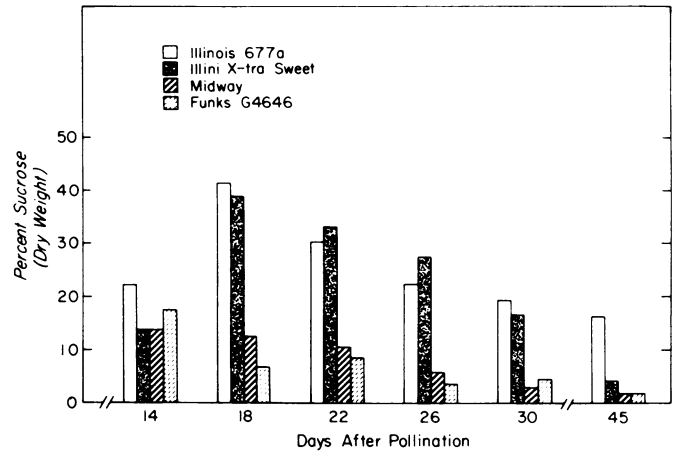


FIG. 3. Sucrose content of maize endosperms expressed as a percentage of dry weight. For genotypes among days after pollination, the $LSD_{(0.05)}$ = 8.69%. For genotypes within days after pollination, the $LSD_{(0.05)}$ = 8.49%.

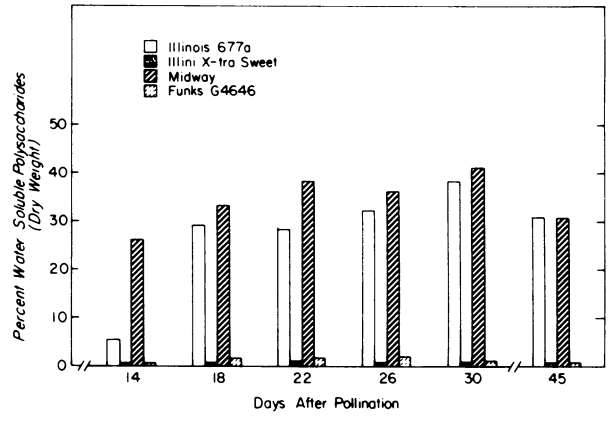


FIG. 4. Phytoglycogen content of maize endosperm expressed as a percentage of dry weight. For genotypes among days after pollination, the $LSD_{(0.05)}$ = 10.40%. For genotypes within days after pollination, the $LSD_{(0.05)}$ = 9.94%.

### MOISTURE

The per cent moisture at six stages of kernel development is shown in Table I. The starchy genotype showed the most rapid decline in moisture, whereas Illinois 677a showed the least overall decline with a conspicuously slow rate from 30 to 45 days after pollination.

At 45 days after pollination, the moisture content of Illinois 677a was about 58% and the kernels were still soft and plump. In contrast, other entries at this stage showed moisture content below 50% and their kernels had begun to harden. This slow drying characteristic of Illinois 677a has not been reported in other *su* genotypes.

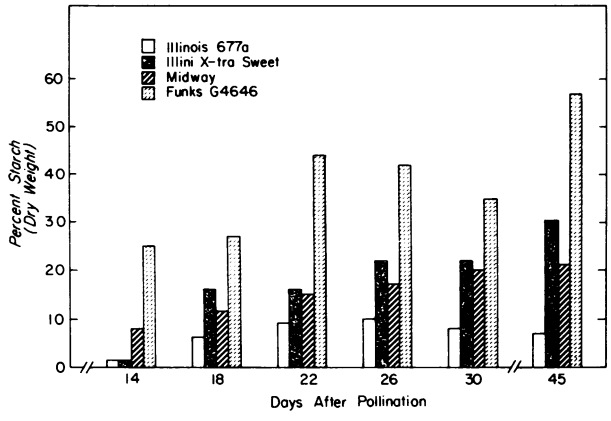


FIG. 5. Starch content of maize endosperm expressed as a percentage of dry weight. For genotypes among days after pollination, the $LSD_{(0.05)}$ = 8.89%. For genotypes within days after pollination, the $LSD_{(0.05)}$ = 8.62%.

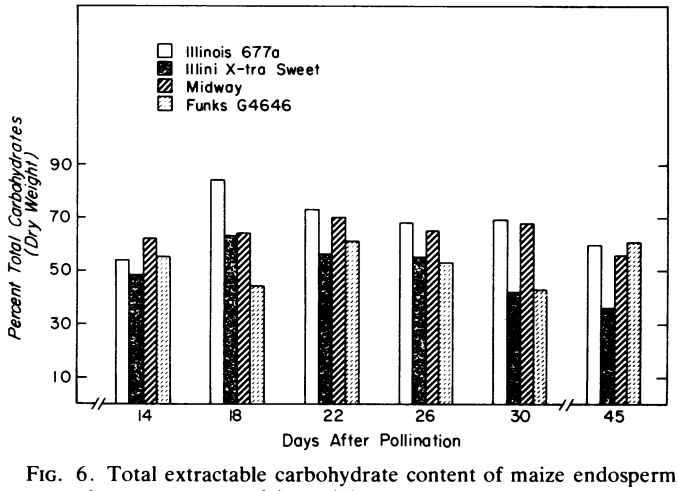


FIG. 6. Total extractable carbohydrate content of maize endosperm expressed as a percentage of dry weight. For genotypes among days after pollination, the $LSD_{(0.05)}$ = 15.43%. For genotypes within days after pollination, the $LSD_{(0.05)}$ = 14.49%.

## PROTEIN AND ENDOSPERM WEIGHT

Average fresh weights of the endosperms were determined, and protein was determined on the various enzyme extracts (Table II). Variation in protein content/endosperm was mostly due to genotypic differences in endosperm weight. Whether significant and perhaps variable amounts of the accumulating reserve proteins were made soluble by the standard enzyme extraction procedure was not known. Therefore, enzyme activity was related to endosperm weight.

## ENZYMES

Increased sucrose accumulation by Illinois 677a endosperm cells might result from enhanced sucrose biosynthesis or reduced hydrolysis. Accordingly, it was checked for a possible reduction of invertase activity and for increases in enzymes (UDP-glucose pyrophosphorylase and sucrose synthase) which are probably responsible for sucrose biosynthesis in maize endosperm. In *sh2*, the drastic reduction in ADP-glucose pyrophosphorylase (6) may indirectly raise the sucrose level, so this enzyme was also assayed in Illinois 677a. Although the *su* enzyme has been studied before (1), the present work is apparently the first to compare the ADP-glucose pyrophosphorylase activity of *su* and starchy.

**Invertase.** Illinois 677a possessed significantly higher soluble and bound invertase than the three hybrids for which carbohydrate data are reported above, and the hybrids did not differ significantly among themselves. Enzyme activities, expressed as nmol sucrose hydrolyzed/min · mg endosperm, for soluble (and bound) invertase were: Illinois 677a, 0.51 (0.12); Midway, 0.11 (0.006); Illini X-tra Sweet, 0.089 (0.012); Funks G4646, 0.046 (0.029). Enzyme activities of the inbred parents of Illinois 677a were: Illinois 44b, 0.13 (0.047); Illinois 442a, 0.26 (0.073). The tendency of sugary genotypes to have more invertase activity than the starchy genotype was also reported by Jaynes and Nelson (14).

**Sucrose Synthase.** No significant differences were found for the genotypes tested. Enzyme activities of Illinois 677a, Funks G4646, Midway, and Illini X-tra Sweet varied over a narrow range, being 0.81 to 1.14 nmol sucrose formed/min · mg endosperm and 0.094 to 0.13 μmol UDP-glucose formed/min · mg endosperm. Illinois 44b and Illinois 442a gave values of 0.12 and 0.16 μmol UDP-glucose formed/min · mg endosperm.

**Sucrose Phosphate Synthase.** Tsai (27) reported a trace (about 1% of sucrose synthase activity) of sucrose phosphate synthase present in developing endosperm of a starchy maize hybrid. Hence, it seemed possible that sucrose accumulation by Illinois 677a could be due to a higher than normal level of this enzyme. However, sucrose phosphate synthase was not detected in the present study. The enzyme was sought in extracts of the four genotypes for which carbohydrate analyses were presented earlier, using crude and dialyzed endosperm extracts at four kernel ages ranging from 14 to 30 days after pollination. Ammonium sulfate fractionation was tried as well as assay of freshly harvested, unfrozen material. It is not clear whether sucrose phosphate synthase was absent from the genotypes tested or whether the assay procedure was not adequate to detect low levels of the enzyme. In either case, these results support the

Table I. *Moisture Content of Maize Endosperm at Various Stages of Kernel Development*

The $LSD_{(0.05)}$ was 6.52% for genotypes among days after pollination and was 6.78% for genotypes within days after pollination.

| Source of Endosperm | Kernel Age Days after Pollination | | | | | |
|---|---|---|---|---|---|---|
| | 14 | 18 | 22 | 26 | 30 | 45 |
| | Moisture % | | | | | |
| Illinois 677a | 83.8 | 71.8 | 69.2 | 65.7 | 63.1 | 58.1 |
| Illini X-tra Sweet | 88.4 | 75.3 | 74.8 | 71.6 | 68.1 | 47.1 |
| Midway | 77.0 | 71.5 | 67.8 | 63.3 | 61.4 | 43.8 |
| Funks G4646 | 82.3 | 71.6 | 62.7 | 53.6 | 46.7 | 30.4 |

Table II. *Soluble Protein and Fresh Weight of Maize Endosperm at 22 Days after Pollination*

Values within a column are not significantly different when followed by the same letter.

| Source of Endosperm | Soluble Protein mg/endosperm | | Fresh Weight mg/endosperm | |
|---|---|---|---|---|
| Illinois 677a | 1.70 | b | 160 | c |
| Illini X-tra Sweet | 2.75 | a | 320 | a |
| Midway | 2.48 | a | 251 | b |
| Funks G4646 | 1.57 | b | 185 | c |
| Illinois 44b | 1.30 | b | 97 | d |
| Illinois 442a | 2.41 | a | 225 | b |

conclusion of earlier authors that sucrose synthase is responsible for sucrose biosynthesis in developing maize endosperm, and sweet corn genotypes, including Illinois 677a, are no exception.

**UDP-Glucose Pyrophosphorylase.** Sucrose accumulation by Illinois 677a over the level found in Midway is apparently not caused by enhanced UDP-glucose pyrophosphorylase. Activities of Illinois 677a, Illini X-tra Sweet, and Midway ranged from 28.3 to 30.8 nmol UTP formed/min·mg endosperm and were not significantly different from each other. Compared to these three, the activity of Funks G4646 was significantly lower (11.2 nmol UTP/min·mg), and the parents of Illinois 677a were significantly higher. Illinois 44b and Illinois 442a gave activities of 44.4 and 40.5, respectively.

**ADP-Glucose Pyrophosphorylase.** Activities of this enzyme, expressed as nmol ATP formed/min·mg endosperm in the presence (and absence) of 12.5 mM 3PGA, were: Illinois 677a, 5.93 (4.86); Funks G4646, 4.32 (2.93); Midway, 3.97 (2.78); Illini X-tra Sweet, 0.20 (0.037). Illinois 677a was not significantly different from Funks G4646. Midway was significantly lower than Illinois 677a, but not different from Funks G4646. As expected, Illini X-tra Sweet was significantly lower than the other entries. Illinois 44b and Illinois 442a exhibited relatively high activities: 5.08 (3.48) for the former and 5 (3.40) for the latter.

## DISCUSSION

The carbohydrate analysis reveals that the new inbred line, Illinois 677a, is a typical *su* genotype in its phytoglycogen content. However, its elevated sucrose content, as much as 40% of dry weight, makes it comparable in that respect to *sh2*. Illinois 677a kernels exhibited a slower rate of moisture loss during maturation than did the other entries, and this trait may be related to its higher level of dissolved carbohydrate. Whether the altered characteristics of this new line are due to a mutant form of *su* or to modifying genes that alter the expression of *su* is not known.

The mechanism responsible for enhanced sucrose accumulation by Illinois 677a has not been established, but it may be quite different from that of *sh2* and *bt2;* ADP-glucose pyrophosphorylase is drastically reduced in developing endosperms of *sh2* and *bt2* (ref. 6 and data in this paper for *sh2*), but the enzyme is not reduced in Illinois 677a compared to a starchy genotype and three *su* genotypes. The *in vitro* activities of invertase, sucrose synthase, and UDP-glucose pyrophosphorylase also do not vary in ways that explain enhanced sucrose accumulation by the new inbred. Altered *in vivo* activity of these enzymes is still a possibility, particularly since activator and inhibitor or inactivator molecules are known for them (5, 12, 13, 15, 27).

The reduced starch content of the new inbred may be related to its unique ability to accumulate phytoglycogen and a high level of sucrose simultaneously. Whether high sucrose and phytoglycogen might be the cause or the result of a reduction in starch is not yet clear. The more general question of how *su* varieties differ metabolically from starchy maize is also not resolved.

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