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# Influence of Gene Dosage on Carbohydrate Synthesis and Enzymatic Activities in Endosperm of Starch-Deficient Mutants of Maize

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**In cereals, starch is synthesized in endosperm cells, which have a ploidy level of three. By studying the allelic dosage of mutants affecting starch formation in maize (*Zea mays* L.) kernels, we determined the effect of down-regulated enzyme activity on starch accumulation and the activity of associated enzymes of carbohydrate metabolism. We found a direct relationship between the amount of starch produced in the endosperm and the gene dosage of *amylose extender-1*, *brittle-2*, *shrunken1*, and *sugary-1* mutant alleles. Changes in starch content were found to be caused by changes in the duration as well as the rate of starch synthesis, depending on the mutant. Branching enzyme, ADP-glucose pyrophosphorylase, and sucrose synthase activities were linearly reduced in endosperm containing increasing dosages of *amylose extender-1*, *brittle-2*, and *shrunken-1* alleles, respectively. Debranching enzyme activity declined only in the presence of two or three copies of *sugary-1*. No enzyme-dosage relationship occurred with the *dull1* mutant allele. All mutants except *sugary-1* displayed large increases (approximately 2- to 5-fold) in activity among various enzymes unrelated to the structural gene. This occurred in homozygous recessive genotypes, as did elevated concentrations of soluble sugars, and differed in magnitude and distribution among enzymes according to the particular mutation.**

Starch, the principal constituent of maize (*Zea mays* L.) grain, is important for its many uses as a foodstuff and as a source of industrially derived products. Mutants displaying altered phenotypes of seed starch deposition have been important in advancing our understanding of how starch is produced in plants. Numerous starch mutants have been reported for maize (Glover and Mertz, 1987; Coe et al., 1988), and several (e.g. *ae1*, *sh2*, and *su1*) have been described extensively for their effects on carbohydrate composition and response to genetic background, allelic dosage, or interaction with other mutations (Creech, 1965; Holder et al., 1974; Garwood et al., 1976; Garwood and Vanderslice, 1982). Many studies of starch mutants have focused on the molecular structure of synthesized polysaccharides and on the concentration and type of soluble carbohydrates found in the kernel during early-to-mid-development. These studies provide a wealth of information about the genetic control of carbohydrate composition in maize grains, but they typically do not describe how these genes affect the developmental pattern and final amount of starch deposited in the seed.

Maize starch mutants have also been instrumental in studying carbohydrate metabolism in seeds and identifying those enzymes or proteins that function in starch biosynthesis (for reviews, see Shannon and Garwood, 1984; Boyer, 1985; Boyer and Hannah, 1994; Nelson and Pan, 1995). Examples of such mutants are *bt1*, *bt2*, *sh2*, and *su1*, which seriously affect kernel dry matter accumulation (i.e. starch deposition), and *ae1*, *du1*, and *sh1*, which produce more moderate phenotypes (Glover and Metz, 1987). The enzymatic reaction or protein affected by these genes has been identified in many cases: *ae1* controls an isoform of BE (Hedman and Boyer, 1982), *sh1* controls SS-I (Gupta et al., 1988), *bt2* and *sh2* control different subunits of AGP-ase (Hannah and Nelson, 1975), *su1* controls a De-BE (Pan and Nelson, 1984; James et al., 1995), and *bt1* corresponds to an amyloplast membrane protein (Cao et al., 1995; Sullivan and Kaneko, 1995).

Several studies have demonstrated that mutant alleles affecting the starch pathway not only have site-specific effects on metabolism but also influence nontarget enzymes. For instance, in maize the activities of AGP-ase in *ae1* and PFK in *sh1* mutants increase compared with wild-type kernels (Ozbun et al., 1973; Doehlert and Kuo, 1990). In pea seed there is a small but significant increase in SS activity when AGP-ase activity is dramatically reduced by the *rb* mutation (Smith et al., 1989; Rochat et al., 1995). A similar mechanism has been observed in antisensed plants in the form of a large increase in invertase and GK activities when SS activity was reduced in potato tubers (Zrenner et al., 1995). Work by Doehlert and Kuo (1994) and Giroux et al. (1994) has shown that transcriptional regulation of several genes related to starch and zein biosyntheses are

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Abbreviations: ADPG-PP, adenosine diphosphate-Glc pyrophosphorylase; *ae1*, *amylose extender*; AGP-ase, adenosine diphosphate-Glc pyrophosphorylase; BE, branching enzyme; BSS, bound starch synthase; *bt1*, *brittle*; DAP, days after pollination; De-BE, debranching enzyme; *du1*, *dull1*; FK, fructokinase; GK, glucokinase; PFK, ATP-linked phosphofructokinase; PFP, pyrophosphate-linked phosphofructokinase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; *sh1*, *shrunken*; SS, Suc synthase; SSS, soluble starch synthase; *su1*, *sugary1*; UDPG-PP, UDP-Glc PPi.

strongly affected in endosperm afflicted with mutations that reduce deposition of these materials. It appears that broad changes in storage product metabolism may be commonly induced by specific genetic lesions. The starch mutants, being diverse and well defined in genetics and biochemistry, could be useful in further defining such metabolic changes and uncovering possible interrelationships within starch metabolism.

The gene-enzyme relationships examined in this study have long been known to affect carbohydrate synthesis in maize kernels (Hannah and Nelson, 1975; Chourey and Nelson, 1976; Hedman and Boyer, 1982; Pan and Nelson, 1984). However, to our knowledge, there are no reports relating the degree of enzyme reduction to polysaccharide synthesis rate and duration. By measuring enzyme activities and starch and phytoglycogen content throughout development, we were able to examine these relationships and determine how general metabolism of starch deposition is affected. We used allelic dosage studies of several starch mutants to demonstrate that apportioned degrees of enzymatic capacity correlate with endosperm starch production. We also found that site-specific lesions in carbohydrate metabolism dramatically affect related enzyme activities of the same pathway.

## MATERIALS AND METHODS

### Plant Material and Growth

Maize (*Zea mays* L.) genotypes included in the study were inbred W64A (wild type) and the isogenic, homozygous mutants *bt2*, *du1*, and *sh1*, and inbred A636 (wild type) and the isogenic mutants *ae1* and *su1*. Isogenicity of the materials within the respective inbred backgrounds was evaluated by scoring the isoform pattern of 10 enzymes from 20 seeds of each genotype (Stuber et al., 1988). No differences were detected in banding patterns between the mutants and their corresponding wild-type, inbred lines.

Plants of all genotypes except *bt2* were field-grown on the ICI Seeds research farm located in Slater, Iowa. Plants in the *Bt2/bt2* allelic dosage series were grown in a growth chamber set for a 15-h photo/thermal period, with a PAR of approximately 680 $\mu$E $m^{-2}$ $s^{-1}$ at plant ear height and a diurnal ramping of temperature between 20 and 25°C.

In establishing allelic dosage series, one dose of the triploid endosperm is contributed by the male and two by the female. Hence, 0, 1, 2, and 3 mutant alleles were obtained by self-pollinating the wild-type genotype, making reciprocal pollinations between the wild-type and mutant genotypes, and self-pollinating plants containing the mutation, respectively. A separate experiment with a complete series of allelic doses was established for each starch-deficient mutation.

Developing kernels were harvested at intervals between 17 DAP and maturity. Black layer formation (Daynard and Duncan, 1969) occurred in kernels of all groups by the final harvest. Samples of 10 to 20 kernels were removed from single ears for each sampling as described by Duncan and Hatfield (1964). The kernels were frozen with dry ice before being transported to the laboratory and subsequently stored at −80°C. To minimize fungal infection, sampled ears were sprayed with a solution of benomyl (0.373 g/L; methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate) before the husks were replaced. The endosperm was dissected from harvested kernels (and the pericarp and embryo discarded) and weighed following lyophilization. Dried endosperm was ground with liquid N to a powder and returned to −80°C storage.

The rate of starch synthesis was taken as the slope of endosperm starch content regressed over the first two harvest intervals (17–31 DAP) of kernel growth. The coefficient of determination was greater than 0.98 in each case. Because the grain-filling period was shortened by the *bt2* gene, the rate was calculated where possible using the first harvest interval (20–27 DAP) for this mutant. Duration was calculated as the quotient of the average endosperm starch content (Table I) and the rate of starch synthesis (Table II).

### Endosperm Analyses

Enzymes were extracted from lyophilized endosperm tissue (100 mg) by homogenization for 20 s (Virtishear homogenizer, Virtis Company, Gardner, NY, 25,000 rpm) at 4°C with 2 mL of extraction buffer (50 mM Hepes-NaOH, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 1 mg/mL BSA). The homogenate was centrifuged (30,000*g*, 20 min, 4°C) and the supernatant saved for assay of soluble enzymes. The pellet was washed twice with extraction buffer (centrifugation as above) and resuspended in 1 mL of the same buffer to obtain BSS. No effort was made to remove phytoglycogen from extracts of *su1/su1/su1* endosperm. The activities of FK, GK, SS, and De-BE were measured in dialyzed extracts, with dialysis conducted overnight at 4°C in extraction buffer. In all cases, each extract was assayed at least twice. One unit of enzyme activity was defined as 1 $\mu$mol of product produced per min at 25°C. Furthermore, all assays displayed linear reaction rates with time and amount of protein added.

De-BE (amylopectin 6-glucanhydrolase, EC 3.2.1.41, also known as pullulanase) was assayed by measuring the amount of pullulan hydrolysis occurring after 0, 30, and 60 min (Doehlert and Knutson, 1991).

Assays used to measure SS (UDP-Glc:D-Fru 2-glucosyltransferase, EC 2.4.1.13), hexokinases (ribonucleotide triphosphate:D-hexose 6-phosphotransferase, EC 2.7.1.1), PFP (PPi:D-Fru-6-P 1-phosphotransferase, EC 2.7.1.90), PFK (ATP:D-Fru-6-P 1-phosphotransferase, EC 2.7.1.11), and AGP-ase (ATP:D-Glc-1-P adenyltransferase, EC 2.7.7.27) have been previously described by Singletary and Below (1990). PGM (D-Glc-1,6-bisphosphate:D-Glc-1-P phosphotransferase, EC 2.7.5.1), PGI (D-Glc-6-P ketoisomerase, EC 5.3.1.9), and UDPG-PP (UTP:D-Glc-1-P uridyltransferase, EC 2.7.7.9) were assayed by the procedures of Doehlert et al. (1988), except that 1 mM UDP-Glc, 50 $\mu$M Glc-1,6-bisphosphate, and 2.5 units/mL of the coupling enzymes was used with the latter enzyme. Except for SS, these enzyme activities were measured by mixing reactions in a microtiter plate and monitoring the change in $A_{340}$ with an automated microplate reader (model EL340, BIO-TEK In-

struments, Winooski, VT). To minimize variability between triplicate assays run in microtiter wells, Tween 20 (0.002%, v/v) was included in the reaction mixtures.

SSS (ADP-Glc:1,4-α-glucan 4-glucosyltransferase. EC 2.4.1.21) assays contained 100 mM Bicine (pH 8.3), 4.5 mM EDTA, 25 mM KCl, 10 mM GSH, 1 mM [$^{14}$C]ADP-Glc (222 dpm/nmol), 2 mg of rabbit liver glycogen, and 50 μL of enzyme extract in a final volume of 0.2 mL. The reaction was stopped with 0.1 mL of 0.25 N NaOH after 10 min for complete reactions and at zero time for controls. Glucan was precipitated by adding 1 mL of methanol and chilling the mixture for 5 min. After centrifugation (12,400*g*, 5 min, 4°C) and removal of the supernatant, the pellet was redissolved in 0.3 mL of 0.1 N NaOH. Alcohol precipitation and dissolution in NaOH was repeated twice again to remove unused radiolabeled substrate. After the final wash, the glycogen pellet was dissolved in 0.1 mL of 1 N HCl, boiled for 10 min, cooled, and supplemented with 1 mL of scintillation cocktail.

BSS assays were carried out in a manner similar to those of SSS, except the enzyme extract consisted of resuspended endosperm tissue prepared as described above. In addition, glycogen was omitted from the assay, which was run for 20 min, and the reaction contained 7.5 mM [$^{14}$C]ADP-Glc (59.2 dpm/nmol). After the starch pellet was boiled in 1 mL of 1 N HCl, 0.9 mL of the solution was transferred to a scintillation vial, 10 mL of cocktail was added, and radioactivity was counted.

BE (1,4-α-D-glucan:1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase, EC 2.4.1.18) activity was measured as the stimulation of 1,4-α-glucan formation from [$^{14}$C]Glc-1-P catalyzed by rabbit muscle phosphorylase *a*. The reaction mixture contained 50 mM sodium citrate (pH 6.0), 1 mM AMP, 2.5 units/mL phosphorylase *a*, 120 mM [$^{14}$C]Glc-1-P (2.22 dpm/nmol), and 50 μL of enzyme extract in a final volume of 0.2 mL. As with the SSS assay, after 10 min reactions were stopped with NaOH and 2 mg (80 mg/mL) of carrier glycogen was added. Subsequent isolation of radiolabeled glucan followed the procedures described for SSS. Assays stopped with NaOH at time zero or those lacking BE served as controls.

Starch and phytoglycogen were measured in duplicate 25-mg samples of lyophilized endosperm tissue. Free sugars were first removed by extracting the tissue twice with 2 mL of 80% aqueous ethanol at 60°C for 30 min. Insoluble materials were collected by centrifugation at 2000*g* for 10 min. After sugars were removed, water-soluble polysaccharides (referred to throughout as phytoglycogens) were collected by extracting the pellet twice with 2 mL of water at 25°C for 30 min. After the samples were centrifuged as above, supernatants containing phytoglycogen were collected, combined, and made up to 25 mL with 50 mM sodium acetate (pH 4.5). The pellet remaining after extraction of phytoglycogen was suspended in 25 mL of the same acetate buffer and autoclaved at 121°C for 30 min. Phytoglycogen and gelatinized starch were hydrolyzed to Glc by incubation with amyloglucosidase (12 units) and α-amylase (35 units) at 55°C for 60 min. Glc was quantitated according to the method of Jones et al. (1977). Soluble sugars were measured in a 50-mg sample of lyophilized endosperm tissue by the method of Heberer et al. (1985).

### Statistical Analyses

Each allelic dosage series was repeated four times, with single ears serving as experimental units. Significant effects were judged by analysis of variance, and comparison of

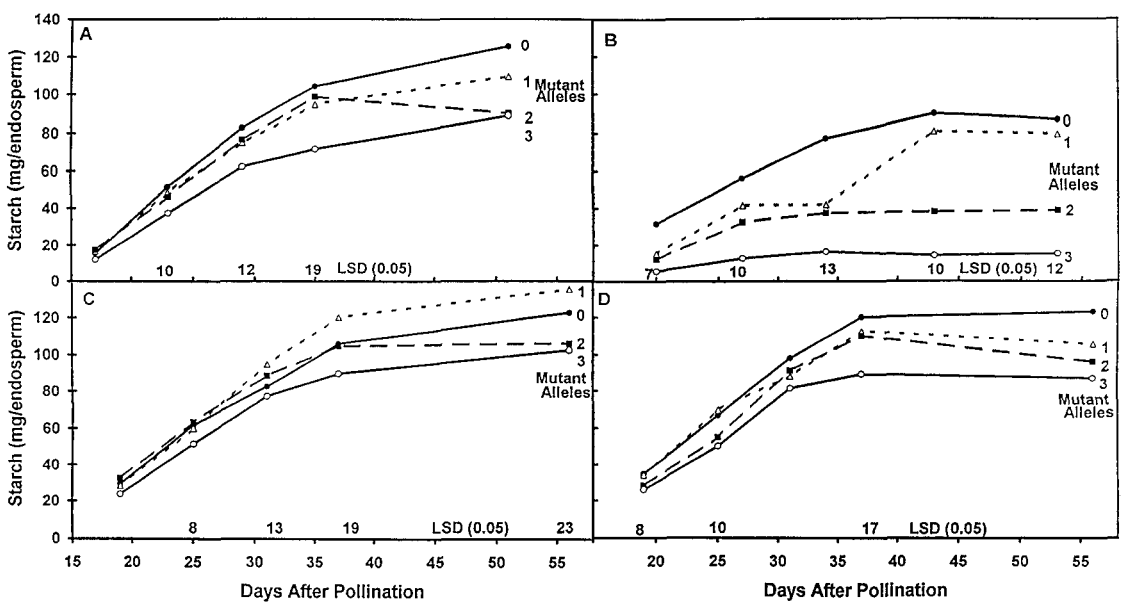


**Figure 1.** Accumulation of starch during the development of maize endosperm containing zero (wild type) to three (mutant genotype) doses of the *ae1* (A), *bt2* (B), *du1* (C), or *sh1* (D) mutant allele. Values are the means of four replications, each representing a single ear.

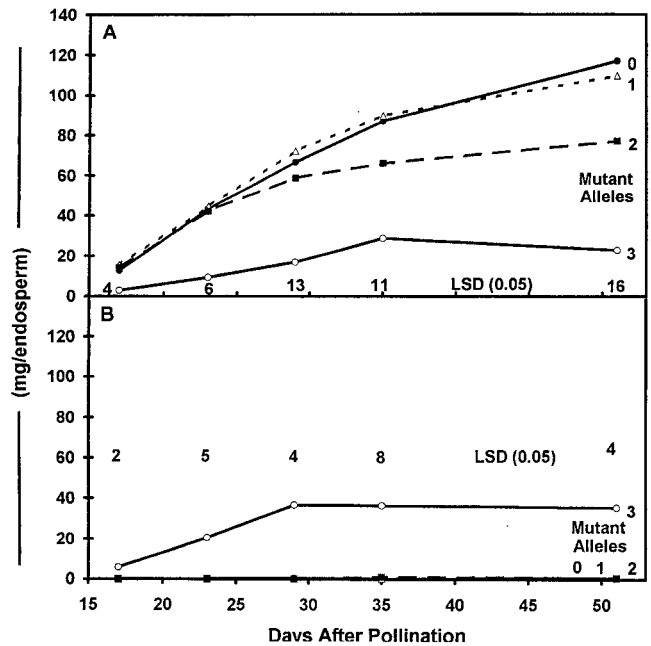


**Figure 2.** Accumulation of starch (A) and phytoglycogen (B) during the development of maize endosperm containing zero (wild type) to three (mutant genotype) doses of the *su1* mutant allele. Phytoglycogen was obtained from endosperm tissue after first removing free sugars and subsequently extracting twice with water. Values are the means of four replications, each representing a single ear.

means was performed by calculating the LSD. Significance was defined as $P < 0.05$ for all tests.

## RESULTS

### Carbohydrate Accumulation

Endosperm starch levels during development of the various genotypes are presented in Figures 1 and 2A. In general, starch content increased during development in a linear fashion until reaching a stationary phase of maximal content. One exception to this pattern occurred in endosperm containing three copies of *bt2*, in which grain-filling duration was noticeably shortened and a delineated period of linear starch accumulation was absent (Fig. 1B). The accumulation profile of phytoglycogen, a glucan similar to starch but more highly branched, was examined in the *su1* gene-dosage series (Fig. 2B). Phytoglycogen was detectable only in *su1/su1/su1* endosperm, where it accumulated in a linear manner at approximately twice the rate of starch.

Because of variability among samples collected at the final harvest, comparisons relating gene dosage to final starch accumulation were made using the average endosperm starch content of the last two harvest dates (Table I). The *ae1* gene reduced average endosperm starch content approximately 10% with each increment in copy number, for a maximum loss of 30% compared with wild-type kernels. The largest reduction in starch synthesis occurred within the *bt2* series. Starch declined modestly with one dose of *bt2* but quite sharply with additional doses, leading to a decrease of 83% in *bt2/bt2/bt2* endosperm. Compared with control kernels, starch content increased with one dose of *du1* but was reduced 16% in *du1/du1/du1* endosperm. For *sh1*, starch content declined an average of 9% per dose. Finally, one dose of *su1* had no effect on starch content, but two doses reduced starch 29% and three doses reduced it 77% compared with wild-type kernels. When phytoglycogen and starch are considered together, total glucan content was reduced 43% in endosperm homozygous for *su1* (Fig. 2). It is important to note that among plants in the W64A inbred background, seed growth in the *Bt2/bt2* series, in which plants were grown in a growth chamber, was reduced compared with the *Du1/du1* and *Sh1/sh1* series, in which plants were field-grown (compare wild-type plants in Tables I and II).

The effect of the mutant genes and their dosage on the dynamics of starch (or starch and phytoglycogen, i.e. glucan) synthesis was examined by calculating the rate and duration of starch accumulation (Table II). The *du1* and *sh1* genes did not produce any large or consistent changes in the rate of starch accumulation. The *ae1* and *su1* genes significantly reduced the rate of starch or glucan deposition 26 to 29% in homozygous recessive endosperm, but there was no clear pattern of rate change across the gene-dosage series. For *bt2*, a significant decline in the rate of starch accumulation occurred only in homozygous recessive endosperm, and it is probable that this change was falsely low because of an inability to accurately determine when starch ceased to accumulate.

The duration of starch synthesis was only significantly affected by the *bt2* allele (Table II). Duration was reduced by almost half in *Bt2/bt2/bt2* endosperm compared with controls, and by 20 DAP, starch had nearly ceased to accumulate in *bt2/bt2/bt2* endosperm. There were also indications that other alleles reduced the duration of starch and glucan accumulation (e.g. *du1*, *sh1*, and *su1*), although these changes were not statistically significant.

The endosperm concentration of soluble sugars was measured in select mutant series. Soluble sugar concentra-

**Table I.** *Starch and glucan contents of mature endosperm containing zero, one, two, or three doses of various recessive alleles that interfere with normal starch synthesis*

Values are the mean ($n = 4$) endosperm starch contents of the *ae1*, *bt2*, *du1*, and *sh1* gene dose series and glucan (starch plus phytoglycogen) contents of the *su1* series. Samples of the last two harvest dates within each replication were averaged to estimate the starch or glucan contents of mature endosperm.

| Recessive Allele Type | Allelic Dosage | | | | LSD |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | |
| | % of control | | | | |
| *ae1* | 100 (115)[a] | 89 | 82 | 70 | 13 |
| *bt2* | 100 (88) | 93 | 45 | 17 | 7 |
| *du 1* | 100 (114) | 112 | 92 | 84 | 9 |
| *sh 1* | 100 (122) | 90 | 85 | 73 | 16 |
| *su 1* | 100 (102) | 100 | 71 | 57 | 11 |

[a] Values in parentheses are the starch (*ae1*, *bt2*, *du1*, *sh1*) or glucan (*su1*) content, expressed as milligrams per endosperm of wild-type kernels.

**Table II.** *Rate and duration of starch and glucan (starch and phytoglycogen) accumulation in endosperm containing zero, one, two, or three doses of various recessive alleles that interfere with normal starch synthesis*

Values are the means of rate and duration calculated for four independent ears in each gene-dosage treatment. Rate is the linear regression of starch (*ae1*, *bt2*, *du1*, and *sh1*) or glucan (starch plus phytoglycogen; su1) accumulation over time between the first and third harvests for all mutants except *bt2*, in which only the first two sample times were used. Duration was derived by dividing the starch or glucan content of the mature endosperm (Table I) by the rate of deposition.

| Recessive Allele Type | Allelic Dosage | | | | LSD |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | |
| Rate (*mg starch or glucan/endosperm/d*) | | | | | |
| *ae1* | 5.62 | 4.83 | 4.93 | 4.18 | 0.45 |
| *bt2* | 3.49 | 3.66 | 2.89 | 1.09 | 0.75 |
| *du1* | 4.42 | 5.56 | 4.68 | 4.48 | 0.74 |
| *sh1* | 5.25 | 4.48 | 5.22 | 4.60 | NS[b] |
| *su1* | 4.54 | 4.85 | 3.75 | 3.24 | 0.57 |
| *bt2* (Lee and Tsai, 1985) | 6.09 | –[a] | – | 5.83 | |
| *sh2* (Cox and Dickinson, 1973) | 5.36 | – | – | 5.63 | |
| Duration (d) | | | | | |
| *ae1* | 20.4 | 21.1 | 19.0 | 19.2 | NS |
| *bt2* | 25.2 | 22.2 | 13.9 | 13.7 | 6.1 |
| *du1* | 25.9 | 23.1 | 22.4 | 20.8 | NS |
| *sh1* | 23.2 | 24.4 | 19.8 | 19.4 | NS |
| *su1* | 22.5 | 21.0 | 19.3 | 17.9 | NS |
| *bt2* (Lee and Tsai, 1985) | 40 | – | – | 21 | |
| *sh2* (Cox and Dickinson, 1973) | 34 | – | – | 18 | |

[a] –, Parameter not measured; [b]NS, Nonsignificant.

tion in the *ae1*, *du1*, and *sh1* sets was similar throughout development in kernels with 0, 1, or 2 doses of mutant alleles (Fig. 3). In the presence of three doses there was approximately a 3-fold greater concentration of soluble sugar in immature endosperm (17 or 19 DAP). Differences in sugar concentration between homozygous recessive and other endosperm genotypes gradually declined in magnitude throughout development but still remained a significant difference at maturity for the *du1* and *sh1* gene-dosage series. The response of sugar levels to gene dosage of *su1* and *sh2*, the latter a mutation closely related to *bt2*, was reported previously (Holder et al., 1974; Doehlert et al., 1993).

## Enzyme Activities

Changes in enzyme activity (per endosperm) produced by gene dosage are expressed in Figures 4 to 8 as a percentage of change relative to wild-type endosperm. To allow the translation of reported changes into values of activity, we list in Table III the enzymatic activities per endosperm at two stages of development for each inbred used. Activity levels agree with those previously reported for maize kernels (Tsai et al., 1970; Ozbun et al., 1973; Hedman and Boyer, 1983; Doehlert et al., 1988; Doehlert and Kuo, 1990).

Results shown in Figure 4 demonstrate that there was a linear decline in BE activity with increasing dosage of *ae1* at the two stages of development evaluated. Compared with wild type, BE activity was 82% less in *ae1/ae1/ae1* endosperm. Although the mutation itself is specific for an isoform of BE, several other enzymes of carbohydrate metabolism were also affected by the *ae1* allele. The pleiotropic effect occurred primarily in endosperm carrying three doses of *ae1*. For example, at 23 DAP there were large increases (50–250%) in UTP-FK, PFK, PFP, AGP-ase, and BSS activities in *ae1/ae1/ae1* endosperm (Fig. 4A). Six days later, the same enzyme activities were 200 to 450% greater than activities found in endosperm containing the *Ae1* allele (Fig. 4B).

The *bt2* gene produces the small subunit of AGP-ase in maize, which is needed for normal functioning of the heterodimeric enzyme (Hannah and Nelson, 1976; Bae et al., 1990). As expected, we observed a near-linear decline in AGP-ase activity across the *bt2* allelic dose series (Fig. 5). The maximum decrease was 93% and associated with this change was a pleiotropic effect of *bt2* on other enzymes. The perturbation was not present at 20 DAP, but in 27-d-old *bt2/bt2/bt2* seeds, some enzymes (e.g. UTP-FK, PFK, PFP, SSS) had increased more than 4-fold and others (e.g. GK, ATP-FK, PGI, BE) 2-fold compared with wild-type kernels (Fig. 5).

The metabolic lesion produced by *du1* is not understood. However, Boyer and Preiss (1981) reported a large reduction in SSS-II and, to a lesser extent, BE-IIA activities in endosperm homozygous for the *du1* mutation. In the current study, BE and SSS activities in crude extracts showed no decline with *du1* gene dosage (Fig. 6). Nevertheless, in seeds homozygous for *du1* large increases of activity were detected among several enzymes, including AGP-ase, UTP-FK, ATP-FK, PFP, PFK, and BE (Fig. 6).

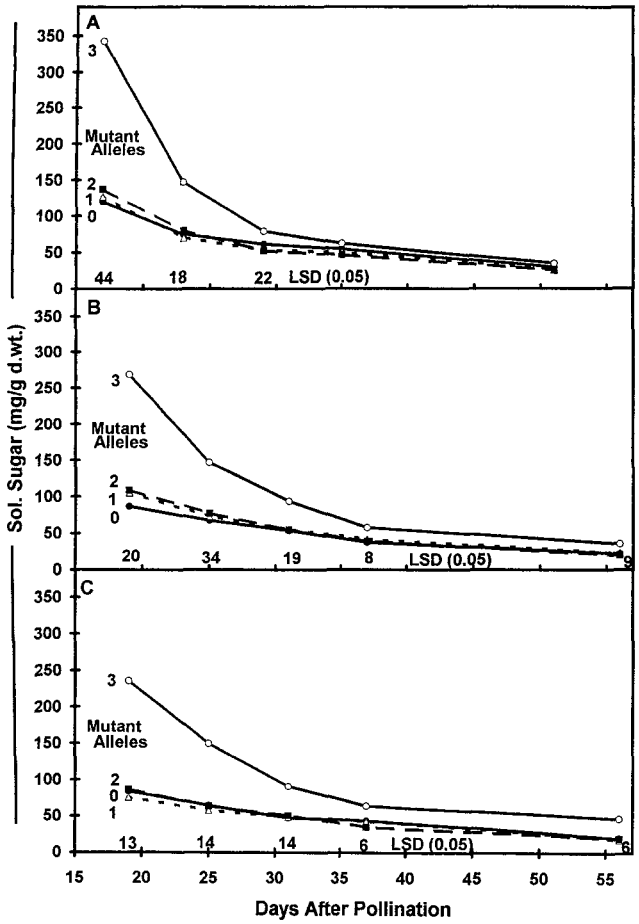


**Figure 3.** The concentration of soluble (sol.) sugars during the development of maize endosperm containing zero (wild type) to three (mutant genotype) doses of the *ae1* (A), *du1* (B), or *sh1* (C) mutant allele. Values are the means of four replications, each representing a single ear. d.wt., Dry weight.

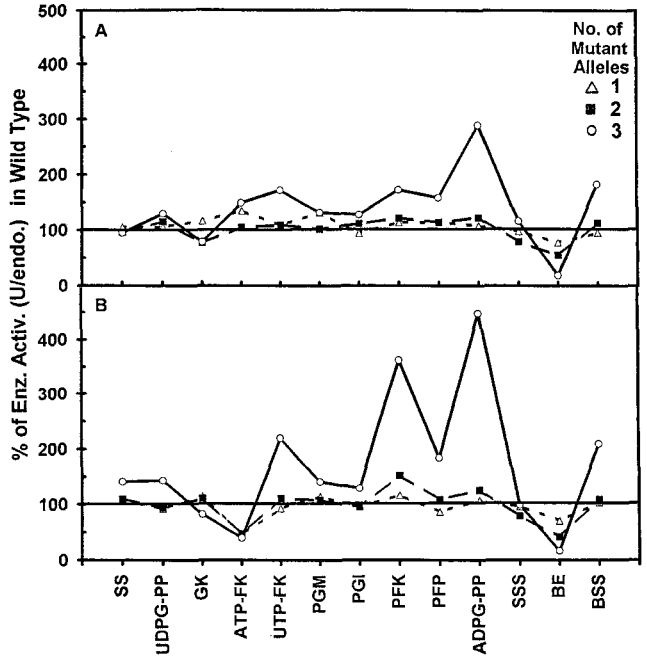


**Figure 4.** Influence of zero (wild type) to three (mutant genotype) doses of the *ae1* mutant allele on the relative activity of carbohydrate metabolism enzymes in endosperm (U/endo.) collected at 23 (A) and 29 (B) DAP. Values are the means of four replications, each representing a single ear. U/endo., Units/endosperm.

The *Sh1* gene encodes the SS-I protein, one of two isozymes of SS present in maize endosperm (Gupta et al., 1988). Like Chourey and Nelson (1976), we also noted a linear decrease in SS activity in the *sh1* dosage series. The maximal reduction was 92% in *sh1/sh1/sh1* endosperm. Akin to most other mutations we tested, there was a strong effect of *sh1* on related carbohydrate-metabolizing enzymes (Fig. 7). The response occurred predominately in the homozygous recessive condition and was most pronounced at 31 DAP (Fig. 7B). Hexokinases, PFK, PFP, AGP-ase, and SSS displayed approximately 200 to 350% more activity per endosperm than was present in the wild type.

The biochemical lesion responsible for the phenotype of *su1/su1/su1* kernels was attributed by Pan and Nelson (1984) to a deficiency in De-BE activity. Recent cloning of the *Su1* gene supports their contention (James et al., 1995) in that the deduced *Su1* polypeptide is most closely related to other enzymes that hydrolyze $\alpha(1\rightarrow6)$ linkages. Pan and Nelson (1984) based their conclusion on chromatographic characterization of separated De-BE activities and a proportional decrease in De-BE activity with increasing numbers of copies of the *su1* allele. In our gene-dose analysis, average De-BE activity (23–29 DAP) was significantly (28 and 65%) reduced with two and three doses, respectively, of the *su1* allele (Fig. 8). One dose had no significant effect on the enzyme and, hence, there was no linear decline of activity with increasing copy number of *su1*. The effect of *su1* dosage on other enzymes was fundamentally different from that seen with other mutants. The *su1* allele decreased, instead of

**Table III.** *Activity of enzymes extracted from wild-type maize endosperm at two stages of development*

Values represent the means of four replications, each constituting a single ear. Extracts were made from endosperm lacking recessive alleles that affect starch accumulation.

| Enzyme | A636 | | W64A | |
|---|---|---|---|---|
| | 23 DAP | 29 DAP | 25 DAP | 31 DAP |
| | *units/endosperm* | | | |
| SS | 0.9 | 1.0 | 1.4 | 1.4 |
| UDPG-PP | 13.6 | 15.5 | 11.8 | 11.0 |
| GK × $10^3$ | 12.1 | 12.3 | 12.6 | 10.8 |
| ATP-FK × $10^3$ | 4.4 | 8.9 | 9.9 | 6.8 |
| UTP-FK × $10^3$ | 13.4 | 9.4 | 8.7 | 3.6 |
| PGM | 3.4 | 5.4 | 3.1 | 2.8 |
| PGI | 0.9 | 1.1 | 0.6 | 0.7 |
| PFK × $10^3$ | 19.0 | 13.1 | 18.6 | 9.8 |
| PFP × $10^2$ | 27.4 | 24.8 | 15.2 | 8.9 |
| AGP-ase × $10^2$ | 20.6 | 14.1 | 14.3 | 13.6 |
| SSS × $10^3$ | 33.3 | 36.2 | 16.6 | 12.0 |
| BE | 1.8 | 1.9 | 1.7 | 1.4 |
| BSS × $10^3$ | 31.0 | 39.8 | 20.0 | 20.8 |
| De-BE × $10^2$ | 2.6 | 3.8 | –[a] | – |

[a] –, Parameter not measured.

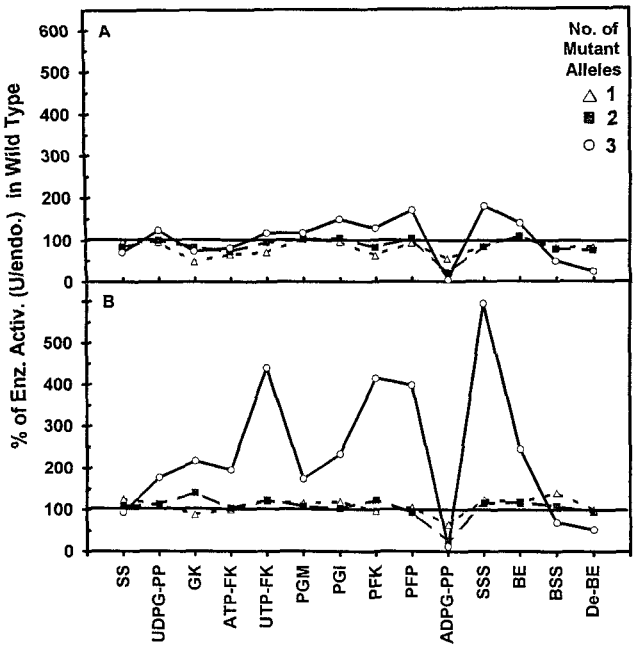


**Figure 5.** Influence of zero (wild type) to three (mutant genotype) doses of the *bt2* mutant allele on the relative activity of carbohydrate metabolism enzymes in endosperm (U/endo.) collected at 20 (A) and 27 (B) DAP. Values are the means of four replications, each representing a single ear. U/endo., Units/endosperm.

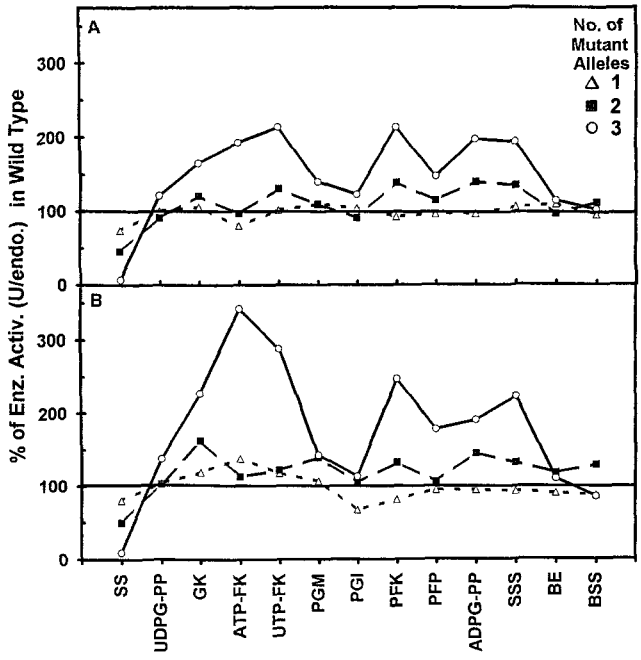


**Figure 7.** Influence of zero (wild type) to three (mutant genotype) doses of the *sh1* mutant allele on the relative activity of carbohydrate metabolism enzymes in endosperm (U/endo.) collected at 25 (A) and 31 (B) DAP. Values are the means of four replications, each representing a single ear. U/endo., Units/endosperm.

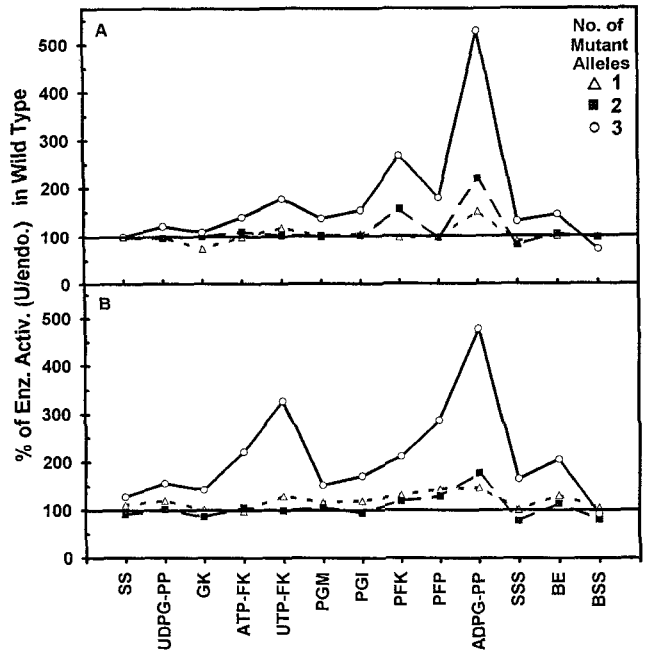


**Figure 6.** Influence of zero (wild type) to three (mutant genotype) doses of the *du1* mutant allele on the relative activity of carbohydrate metabolism enzymes in endosperm (U/endo.) collected at 25 (A) and 31 (B) DAP. Values are the means of four replications, each representing a single ear. U/end., Units/endosperm.

stimulating, the activity of several enzymes of carbohydrate metabolism. In some cases, the response was more pronounced with two rather than three copies of the mutant allele. A reduction (75%) of SSS activity occurred in *su1/su1/su1* endosperm, similar to that observed for De-BE activity, but some of this decline was attributable to the presence of phytoglycogen in the assay (see below). Changes among other enzymes were inconsistent (Fig. 8).

Significant amounts of phytoglycogen occurred in *su1/su1/su1* endosperm and, although it was not removed from our extracts, we considered the possibility that this water-soluble polysaccharide may have interfered with the measurement of enzyme activities. Accordingly, enzyme activities were measured from a co-extract of *Su1/Su1/Su1* and *su1/su1/su1* endosperm and compared with results obtained when the genotypes were assayed independently. With phytoglycogen in the extract, a 10 to 25% overestimation of ATP-FK, PGM, and BE activities and an underestimation of 15% for De-BE and about 40% for AGP-ase and SSS were observed. Doehlert et al. (1993) observed that the addition of purified phytoglycogen to crude extracts from wild-type kernels reduced pullulanase (i.e. De-BE) and α-amylase activities 14 and 75%, respectively.

We applied the flux control theorem (Kacser and Burns, 1973) to our data. This theorem is based on quantifying the fractional change in activity of an enzyme ($\delta E/E$) with the fractional change in flux through a pathway ($\delta J/J$). The flux control coefficient ($C$) is derived by expressing the two fractional changes as a single ratio of $(\delta J/J)/(\delta E/E)$. The equations developed by Torres et al. (1986) were used to estimate the control coefficient: $C = (e - ep)/(p - ep)$,

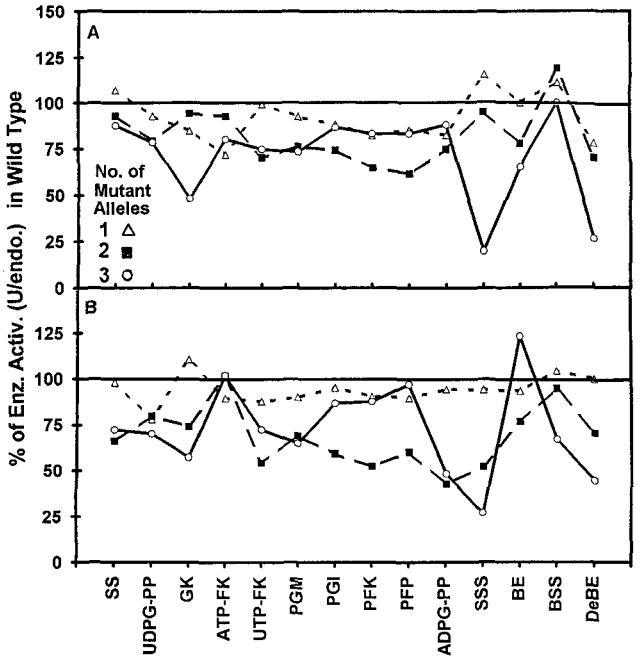


**Figure 8.** Influence of zero (wild type) to three (mutant genotype) doses of the *su1* mutant allele on the relative activity of carbohydrate metabolism enzymes in endosperm (U/endo.) collected at 23 (A) and 29 (B) DAP. Values are the means of four replications, each representing a single ear. U/endo., Units/endosperm.

where $e = E2/E1$ and $P = J2/J1$. Our data (Table IV) show that the flux control coefficients for different enzymes are $C^{AGP\text{-}ase} = 0.04, 0.19$; $C^{BE} = 0.14, 0.07$; and $C^{SS} = 0.01, 0.01$ when comparing two and three doses of the mutant alleles, respectively, with wild-type kernels.

## DISCUSSION

We investigated the biochemical changes associated with the starch mutants *du1*, *sh1*, *ae1*, *su1*, and *bt2* in homozygous and heterozygous gene-dosage combinations. We found a direct relationship between the amount of starch produced in the endosperm and the gene dosage of wild-type alleles of *Ae1*, *Bt2*, *Sh1*, and *Su1* present in the same tissue. The changes in starch content were found to be caused by changes in the duration of starch accumulation, as well as in the rate of starch synthesis, depending on the mutant. Associated with altered storage of starch was an overexpression of other enzyme activities involved in starch deposition, but this primarily occurred in homozygous recessive endosperm coincident with abnormally high concentrations of soluble sugars. The effect of changes in catalytic activity on starch accumulation was not equal among the enzymes. Estimates of flux control strengths show that AGP-ase, BE, and SS share only minimal control of carbon flux into starch in developing maize endosperm. These data are discussed in three parts: (a) overall changes in enzyme activities, (b) specific effects on starch content and rate and duration of starch accumulation, and (c) estimation of control strengths of specific enzymes.

### Overall Changes in Enzyme Activities

Our gene-dosage series provided a stepwise decrease in selected enzyme activities, consistent with earlier reports. At two harvest dates during mid-grain filling, we observed a linear decline in BE, AGP-ase, and SS activities with increasing copy number of *ae1*, *bt2*, and *sh1*, respectively. The *su1* allele has been reported to affect pullulanase activity in a linear gene-dose fashion (Pan and Nelson, 1984). However, in our studies and as noted by Doehlert et al. (1993), pullulanase (i.e. De-BE) activity was reduced by the *su1* allele but not proportionately with *su1* copy number. In view of the recent finding that *Su1* is likely a structural gene for an $\alpha(1\rightarrow6)$ hydrolytic enzyme (e.g. isoamylase, pullulanase, limit dextrinase) (James et al., 1995), we suggest that crude extracts are not appropriate for detecting the activity/dosage relationship that exists for this enzyme.

There was a large pleiotropic effect of the metabolic lesions on other enzymes in the starch synthesis pathway. Thus, several enzymes had higher activities, apparently in response to mutant-specific reductions in key enzymes. These data confirm and extend other reports demonstrating similar responses in maize (Ozbun et al., 1973; Doehlert and Kuo, 1990), exemplifying the pervasiveness of this mechanism among starch-synthesizing enzymes. Giroux et al. (1994) showed that elevated transcription is at least one

**Table IV.** *Control coefficients of starch synthesis in endosperm containing two and three doses of various recessive alleles that interfere with normal starch synthesis*

Values reported are based on equations developed by Torres et al. (1986) and represent the means of four determinations of enzyme activity and flux of carbon into starch synthesis.

| Allele (Enzyme Affected) | $e$[a] | $p$[b] | $e - ep$ | $p - ep$ | Control Coefficient[c] |
|---|---|---|---|---|---|
| Two doses of mutant allele | | | | | |
| *ae1* (BE) | 0.494 | 0.877 | 0.061 | 0.444 | 0.137 |
| *bt2* (AGP-ase) | 0.227 | 0.868 | 0.030 | 0.671 | 0.045 |
| *sh1* (Suc synthase) | 0.468 | 0.994 | 0.003 | 0.529 | 0.005 |
| Three doses of mutant allele | | | | | |
| *ae1* (BE) | 0.170 | 0.744 | 0.044 | 0.617 | 0.071 |
| *bt2* (AGP-ase) | 0.080 | 0.310 | 0.055 | 0.285 | 0.195 |
| *sh1* (Suc synthase) | 0.088 | 0.876 | 0.011 | 0.799 | 0.014 |

[a] $e = E2/E1$. [b] $p = J2/J1$. [c] Control coefficient = $(e - ep)/(p - ep)$.

mechanism by which enhanced enzyme activities can occur in mutant kernels. They also showed that endogenous levels of soluble sugar may function to trigger altered gene expression. In our study we measured soluble sugars in *ae1*, *du1*, and *sh1* endosperm (Fig. 3) and found the concentrations to be higher in kernels containing three copies of mutant alleles. Accordingly, pleiotropic effects on enzyme activities of sugar metabolism occurred primarily in the same mutant endosperm. These observations support the conclusions drawn by Giroux et al. (1994) but do not explain all of our data. For instance, although soluble sugars were increased in *ae1/ae1/ae1*, *du1/du1/du1*, and *sh1/sh1/sh1* endosperm, it was primarily enzymes near the end of the starch pathway that were overexpressed in *ae1* and *du1* mutants (Figs. 4 and 6) and enzymes early in the pathway of sugar metabolism in the *sh1* mutant (Fig. 7). Furthermore, although *su1* mutants are well known for their increased levels of soluble sugars, in our study there was no indication of overexpression in homozygous *su1* endosperm (Fig. 8). Our data for *su1* are similar but not identical to those of Doehlert et al. (1993), who reported no overexpression in many enzymes but some increase in activity of AGP-ase; we detected no increase. Perhaps these minor differences may be explained by differences in the severity of mutant alleles or by differences in mutant backgrounds.

An interesting point concerning the regulation of activities of sugar-metabolizing enzymes is the correlation we observed (Figs. 4–8), which encompasses a wide range of activities in different mutant backgrounds. When we expressed enzyme activities as a percent change relative to the respective wild-type endosperm and compared these changes across all of the mutants evaluated, we identified interrelationships among UTP-FK, UDPG-PP, PGI, PGM, PFP, and PFK activities (Fig. 9). No other enzymes, including SS, demonstrated significant correlations. These data suggest that these enzymes of sugar metabolism share some linked or common features of control of expression.

Finally, these data indicate that the changes in starch fine structure seen with the maize starch mutants may not be linked solely to losses in the activity of particular enzymes caused by the mutation. The full mutants also have significant levels of overexpression of other key synthetic enzymes (Figs. 4–8). Intermediate gene-dosage combinations (e.g. *ae1/ae1/Ae1* or *Ae1/Ae1/ae1*), which also have significant reductions in activity of key enzymes, do not display any significant increases in other enzyme activities and are noteworthy for their lack of change in starch fine structure (data not presented). Thus, we tentatively suggest that the changes in starch fine structure observed with the mutants are a consequence of specific reductions in some enzymes, perhaps in combination with overexpression of other enzymes involved in starch biosynthesis.

### Specific Effects on Starch Content and Rate and Duration of Starch Accumulation

When comparing enzyme levels with final glucan content (i.e. starch or starch plus phytoglycogen), we esti-

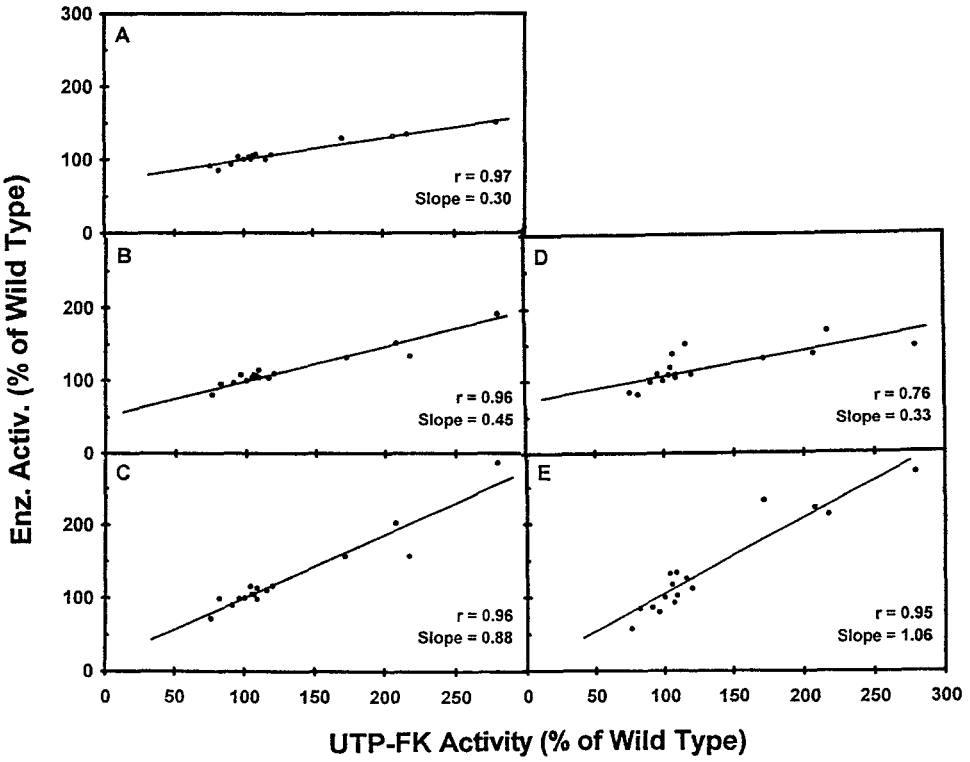


**Figure 9.** Relationship between changes in activity of UTP-FK and changes in activity of UTP-FK and UDPG-PP (A), PGI (B), PFP (C), PGM (D), and PFK (E) as induced by dosage of recessive alleles (*ae1*, *bt2*, *du1*, *sh1*, and *su1*) that interfere with endosperm starch biosynthesis. UTP-FK was arbitrarily chosen as the common regression attribute.

mated that for each 10% loss of BE or SS activity there was a linear reduction in endosperm starch content of 3.5 and 2.8%, respectively (based on slopes, Fig. 10). The best fit of similar data for *bt2* and *su1* occurred with quadratic equations, in which the presence of more than one recessive allele restricted glucan accumulation approximately 30% per dose (Fig. 10). Although *ae1*, *bt2*, *sh1*, and *su1* alleles each depressed starch accumulation in a predictable manner, their actions on the rate and duration of starch deposition were different. Mutations that reduced activities of BE (i.e. *ae1*) and De-BE (i.e. *su1*) appeared to cause a reduction in the rate of starch synthesis, with no detectable effects on duration (Table II). For SS (i.e. *sh1*) there were only small effects on the rate and duration of starch synthesis.

The decrease in final starch content conditioned by dosage of *bt2* appears to be associated with changes in the duration of starch synthesis and may also be linked to changes in starch synthesis rate. Thus, with two doses of *bt2* the resulting lower activity of AGP-ase was associated with reduced endosperm starch content, primarily because of a shorter period of starch production. In the homozygous recessive condition, with three doses starch synthesis duration was again reduced and it seems that the rate of starch synthesis was also impaired (Table II). However, because our analysis for *bt2* does not begin until 20 DAP and because it relies on only two time points, there is the need for caution. We would have underestimated the rate and overestimated the duration if the synthesis rate of starch was already beginning to slow by 20 DAP. When data for early seed development presented by other workers (Cox and Dickinson, 1973; Lee and Tsai, 1985) are used, it is clear that the starch synthesis rate is unaffected in *bt2* and *sh2* mutants compared with wild-type controls (see Table II). Taken together, these data strongly suggest that the decrease in starch content conditioned by reduced AGP-ase activity occurs primarily because of an early termination of starch biosynthesis, perhaps with little contribution by a reduced rate of starch synthesis. It is not clear why reduced AGP-ase activity primarily affects the duration of starch synthesis. However, we hypothesize that in the wild-type condition there is normally an overabundance of AGP-ase activity, such that reductions in activity could occur without detectable changes in flux through the pathway. To explain the early termination of starch synthesis, we suggest that, since enzyme activities are highest early in grain development and then decline as grain filling proceeds, it is possible that the reduced enzyme activity caused by gene dosage of mutant alleles could cause kernels to reach limiting enzymatic capacities earlier in development than is normal.

Finally, it is clear that decreased starch content can occur in heterozygous seeds, even though there is no observable mutant phenotype. This seems to be common among metabolic lesions that only moderately affect starch synthesis (e.g. *ae1*, *du1*, and *sh1*). In contrast, we have observed in repeated greenhouse experiments that mutants such as *bt2* and *sh2*, which dramatically disrupt normal starch production, display a visual decrease in kernel size with two or three doses of the mutant allele.

### Estimation of Control Strengths of Specific Enzymes

One key objective of this study was to determine flux control coefficients (Kacser and Burns, 1973; Small and Kacser, 1993) of several enzymes in the pathway of starch biosynthesis. Control theory (Kacser and Burns, 1973; Torres et al., 1986) dictates that control of flux through a metabolic pathway can be shared between several enzymes or associated with only one enzyme in the pathway. The predominant theory of control over starch synthesis involves the enzyme AGP-ase. Although there has been some confirmation of this in Arabidopsis leaves in low-light conditions (Neuhaus and Stitt, 1990), there are no control strength measurements for AGP-ase in starch-storage tissues. Furthermore, although increased starch content has been reported in potato tubers overexpressing the bacterial enzyme (Stark et al., 1992), there were no actual measurements relating AGP-ase activities to rates of starch synthesis. Thus, claims that these data provide evidence of high levels of flux control by AGP-ase are premature.

It is well known (Boyer and Hannah, 1994) that maize seeds express multiple isozymes for the enzymatic activities we measured for control strength analysis. It is important to note that our enzymatic measurements do not discern differences in activity contributed by individual isozymes. Various isozymes may singularly respond to gene dosage differently than the overall response observed using crude

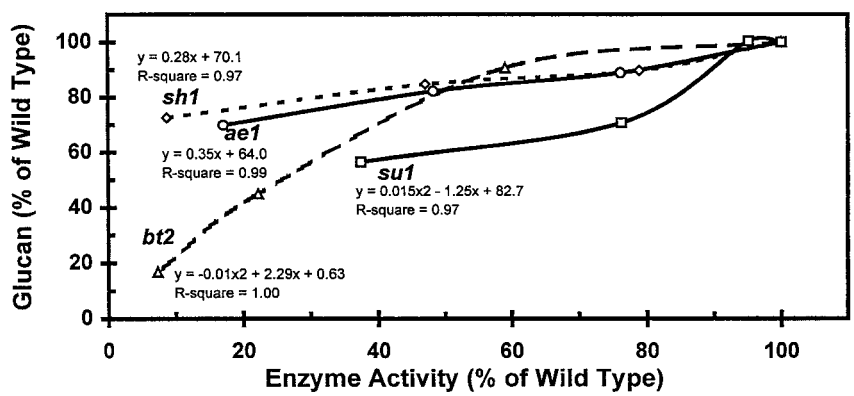


**Figure 10.** Relationship between enzyme activity and glucan content of developing endosperm as influenced by gene dosage. Endosperm contained zero (wild type) to three (mutant genotype) doses of the *ae1*, *bt2*, *sh1*, or *su1* mutant alleles. Glucan was the sum of starch and phytoglycogen for *su1* endosperm, and represents starch only for other genotypes. Values for starch content were taken from Table I. Calculations involving enzyme activity (units/endosperm) utilized a mean of values obtained from three sample dates occurring between 17 and 31 DAP. Enzymes measured were SS, BE, De-BE, and AGP-ase for *sh1*, *ae1*, *su1*, and *bt2*, respectively.

extracts for enzymatic measurement. For our estimation of control coefficients, the enzymatic change is based on the whole of activity measured for a particular catalytic reaction. Hence, applying the equations presented by Torres et al. (1986) to starch synthesis rate for two and three doses of mutant alleles, we calculated flux control strengths of 0.14 and 0.07 for BE in the *ae1* mutant, 0.04 and 0.19 for AGP-ase in the *bt2* mutant, and 0.01 and 0.01 for SS in the *sh1* mutant. Estimates of control strengths for *du1* and *su1* mutants were not possible because either the changes in enzyme or the changes in flux were too small to reliably calculate a ratio. These data from *ae1*, *bt2*, and *sh1* mutants provide evidence that BE, AGP-ase, and SS have only minimal control over starch biosynthesis in developing maize endosperm. Our findings are apparently not consistent with the recent report that a change in allosteric properties of the *shrunken-2* maize AGP-ase polypeptide can increase seed weight by 11 to 18% (Giroux et al., 1996). However, the relationship between this change in seed weight and starch biosynthesis, or seed metabolism in general, is unclear since starch concentration in the seed was unchanged. Presumably, the allosteric variant caused an increase in oil, protein, and/or fiber deposition as well. On the other hand, our findings are in clear agreement with data from pea cotyledons, in which low flux control strengths were reported for AGP-ase and BE (Denyer et al., 1995).

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