CHANGES IN STARCH GRANULE SIZE AND AMYLOSE PERCENTAGE DURING KERNEL DEVELOPMENT IN SEVERAL *ZEA MAYS* L. GENOTYPES[1]

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ABSTRACT

Amylose concentration in starch reportedly increases with increasing physiological age of the tissue in which the starch is synthesized. Analyses of starches from developing maize (*Zea mays* L.) endosperms of two independently occurring *amylose-extender* (*ae*) alleles, named *ae-Ref* (Reference) and *ae-il* (induced-1), the double mutant *amylose-extender sugary (ae su)*, and *normal* were consistent with this developmental pattern; however, the mutant *waxy (wx)* and the double mutant *amylose-extender waxy (ae wx)* were exceptions. The apparent amylose percentage of *wx* remained near zero and that of *ae wx* decreased during kernel development. Maximum starch granule size from all genotypes increased during kernel development from 18 to 36 days post-pollination, but granule size distributions varied among genotypes. *Normal* and *wx* kernels produced the largest granules. Compared to *normal*, the maximum granule size was reduced in *ae-Ref*, *ae-il*, and *ae wx*, resulting in an increased frequency of medium to small granules. Granules from *ae su* kernels generally were very small, although a few granules were as large as those from *normal*. When starch granules from 36-day-old *ae-Ref*, *ae su*, and *normal* kernels were separated into different size classes, a decline in apparent amylose percentage with decreasing granule size was observed. Thus, the smaller granules reflected the characteristics of unfractionated starch isolated from whole endosperms earlier in kernel development, supporting the hypothesis that these small granules were derived from physiologically younger cells.

Endosperm cells of a developing maize (*Zea mays* L.) kernel are comprised of a population of cells of varying physiological ages (1,2,3). The basal endosperm cells begin starch biosynthesis late in kernel development and contain small starch granules (1). Peripheral endosperm cells, which are the last to develop, also contain small starch granules (2). Thus, two gradients of cellular development exist in maize kernels: a major gradient from the central crown part of the kernel to the point of attachment of the kernel, and a minor gradient from the central kernel outward to the peripheral endosperm cells. However, granules within a given cell from *normal* maize kernels are similar in size (3).

Since all endosperm cells are not the same age, the physiologically younger cells may undergo the same developmental changes in starch biosynthesis as older cells but at a later time in kernel development. Shannon (4) subdivided endosperm from 30-day-old *normal* kernels into seven zones from the point of kernel attachment to the crown. Endosperm zones from near the base contained high amounts of soluble sugar and low amounts of starch, similar to the carbohydrate composition reported for whole endosperms 8, 10, and 12 days

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post-pollination (5). The low sugar, high starch content of the upper endosperm zones was similar to the carbohydrate composition of whole endosperms 22 and 28 days post-pollination (5). Furthermore, incorporation of ^{14}C into starch granules per unit granule surface area was similar for all starch granules larger than those containing approximately 0.1 γ of starch per granule (3). Smaller granules contained only half as much ^{14}C per unit granule surface. From these data and cytological observations, it was suggested that the smallest granules may have been derived from physiologically younger cells which had not developed full starch biosynthetic capacity (3).

Another developmental change observed in various plant species has been an increased amylose concentration with increasing age of the tissue from which the starch was isolated. For example, Badenhuizen and Chandorkar (6) found that the amylose content of tobacco-leaf starches increased as older leaves were sampled. Similarly, wheat-kernel starch showed an increased amylose percentage from 2 to 6 weeks after anthesis (7), and Banks *et al.* (8) found a doubling in amylose percentage in barley kernel starch from 9 to 46 days after anthesis. Increasing amylose concentrations were similarly observed in both *normal* and amylomaize (hybrids with the *amylose-extender* gene) starches (6,9) isolated from kernels of advancing development. However, developmental changes in amylose content for other maize endosperm mutants which differ markedly in starch properties (10) have not been evaluated.

If physiologically younger cells lack full biosynthetic capacity as suggested previously (3), amylose concentration in starch from older endosperms would be predicted to vary as a function of granule size. To test this hypothesis and evaluate changes in amylose percentage during development of additional maize mutants, the patterns of amylose accumulation were investigated for six maize genotypes, and the amylose content was determined in different starch granule size classes separated from endosperms collected 36 days post-pollination.

MATERIALS AND METHODS

Genetic Material

The dent maize inbred W64A (normal); two *amylose-extender* alleles, named *ae-Ref* and *ae-il*, which have different amylose contents at maturity (11); *waxy (wx)*; and the double mutants *amylose-extender sugary (ae su)* and *amylose-extender waxy (ae wx)* were used. These genotypes had been backcrossed to W64A 9, 6, 8, 7, and 4 times, respectively. The *ae su* and *ae wx* double mutants contained the *ae-Ref* allele. *Normal* contained the dominant alleles at these gene loci and thus had the genotype *Ae Ae Su Su Wx Wx*. The plant materials were grown in 1972 at The Pennsylvania State University Rock Springs Agricultural Research Center in a split-plot design. The post-pollination dates of harvest were used as the main plots, and genotypes were used as the subplots. Two ears per genotype were sampled from each of two replications by date. All plants were self-pollinated. Ears were harvested at 18, 24, and 36 days after pollination and frozen in ethanol cooled with Dry Ice within 5 min after removal from the plants. Subsequent storage was at −25°C. Entire ears were freeze-dried and samples of 50, 30, and 20 kernels were removed from the central portion of ears 18, 24, and 36 days post-pollination, respectively.

Endosperms free of pericarp and embryo tissues were soaked overnight in 60

ml of "steep" solution (0.01*M* $HgCl_2$ and 0.02*M* sodium acetate, pH 6.5) in a 40°C water bath. The softened endosperm tissue was ground by means of a porcelain mortar and pestle. The slurry was filtered through Nitex (Tobler, Ernst and Traber, Inc., New York, N.Y.) nylon bolting cloth with sieve openings of 80 μ, and released starch granules were washed through the screen with water. The fibrous material not passing through the screen was returned to the mortar, and the grinding and filtration processes were repeated until essentially all granules were removed from the fibrous material.

Starch granules passing through the 80-μ mesh screen were suspended in a 0.05*M* NaCl solution and purified by shaking repeatedly for 1 hr each time with toluene (12). After centrifugation (1000 × *g*, 15 min) most of the starch was pelleted, while denatured protein plus some starch was located at the interface of the toluene and saline solutions. The material located in the first two interfaces was collected and combined. The trapped starch granules were released by gentle shaking for 1 hr, followed by centrifugation (1000 × *g*, 10 min). Recovered granules were combined with the pelleted granules. Since the bulk of the protein was removed with the first two toluene extractions, very little starch was trapped in the subsequent toluene washes. Thus, no further attempts were made to recover the few granules which may have been at the interface. Seven toluene extractions for 18-day-old kernels and ten extractions for 24- and 36-day-old kernels gave a clear toluene-saline interface by the final shaking. After the final shaking, the purified starch granules were washed four times with water by suspension and centrifugation as described above, and stored under toluene at 4°C. Starch preparations were checked for purity and freedom from starch granule clumping by light microscopy.

Starch Granule Size Distributions

Size distributions were generated on a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) which was standardized by using synthetic latex beads with a reported diameter of 3.49 μ, and by using paper mulberry pollen (*Broussonetia papyrifera* L., Vent.) with a reported diameter of 12 to 13 μ. The number of granules having diameters from 1.5 to 2.5 μ, from 2.5 to 3.5 μ, etc., were determined, and these values used to calculate the points plotted at 2 μ, 3 μ, etc., respectively, on the granule size distribution figures. Points were generated every 1 μ from 2 μ upward until a maximum size was determined. A size distribution was determined on a single sample from the two ears in the first replication for each genotype at each age. Granule sizes are presented as percentages of the total number of granules counted. Over 50,000 granules were counted for each distribution (Table I).

Separation of Starch Granules by Size

Starch samples from kernels 36 days post-pollination from a single ear of the genotypes *ae-Ref, ae su, ae wx*, and *normal* were washed with acetone, dried, and gently powdered with a mortar and pestle. Starch granules were separated by sifting through sieves with pores of 20, 10, and 5 μ into sizes ranging from 20 to 10 μ, 10 to 5 μ, and smaller than 5 μ. An ATM Allen Bradley Sonic Sifter (Fisher Scientific, Pittsburgh, Pa.) was used. Approximately 100 mg of *ae-Ref* and *ae su* granules passing through the 5-μ sieve were further separated by settling through an aqueous glycerol mixture. These granules were suspended in 20 ml of distilled

water, carefully layered onto 30 ml of 30% glycerol in a 3 × 20-cm glass test tube, covered, and placed in the cold (0°–5° C) overnight. The next day the upper 30 ml was collected as fraction C-1 and the middle 10 ml as C-2. The remaining 10 ml was centrifuged, the supernatant discarded, the starch pellet suspended in 20 ml of distilled water, and the settling procedure repeated. The upper 30 ml of this settling was combined with C-1, the middle 10 ml with C-2, and the bottom 10 ml was collected as C-3.

Determination of Amylose Percentage

Apparent amylose percentages of dimethyl sulfoxide dispersed starch samples were determined by the blue value method of Wolf *et al.* (13) except that the quantity of KIO_3 was increased as previously described (11), and starch concentrations were determined by the phenol sulfuric acid test (14). Duplicate kernel samples from each ear were analyzed. All amylose percentage determinations were done in duplicate.

Statistical Analysis

Analyses of variance were performed using standard techniques (15). Waller

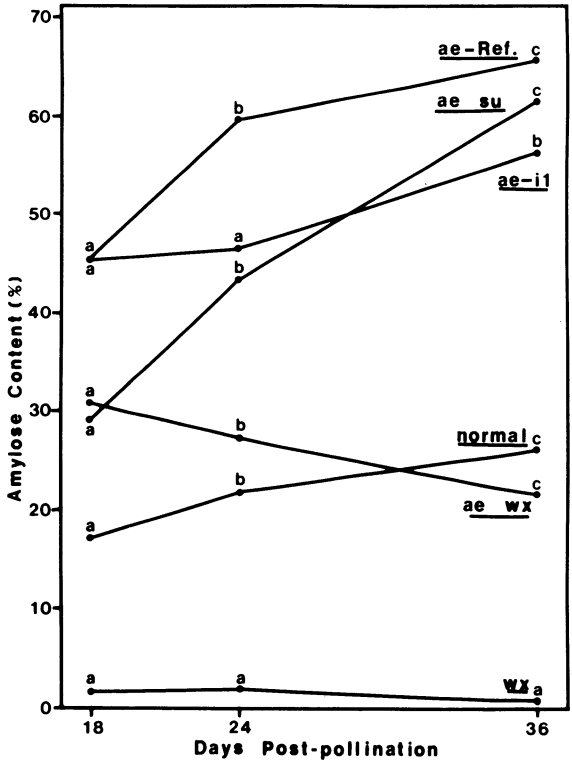

Fig. 1. Changes in amylose content (%) in endosperm starches of six maize genotypes during development. Points on the same line marked by the same letter are not significantly different (k = 100).

and Duncan's Modified (Bayesian) Least Significant Difference Test (16) with $k = 100$ was used to determine mean separations.

RESULTS AND DISCUSSION

The apparent amylose percentages in starch from *normal, ae-Ref, ae-il,* and *ae-su* increased with increasing kernel age (Fig. 1) similar to the amylose changes in *normal* and *ae* kernels reported earlier (6,9). However, that from *ae wx* decreased. Starch from *wx* contained little amylose (approximately 1%) and did not change with increasing age. The rate of change in apparent amylose percentage with age varied with the genotype, and the double mutant *ae su* produced the greatest change (Fig. 1).

The two *ae* alleles had the same amylose percentage at 18 days, but differed after that time (Fig. 1). However, the rate of change for the two alleles from 24 to 36 days post-pollination was very similar, indicating that the difference noted in the mature starches (11) probably occurred between 18 and 24 days after pollination. Comparison of the amylose percentages of *ae-Ref* and *ae su* indicated that the *su* allele in combination with *ae* inhibited the *ae*-enhanced accumulation during early kernel development (up to 18 days), but that after that time the *ae* allele overcame the *su* effect and the percentage of amylose increased at a rate greater than that observed in the single mutant *ae-Ref.*

Starch of *ae wx* showed an unusual pattern of amylose change with increasing age (Fig. 1). As noted above, the *wx* allele effectively blocked amylose accumulation at all sampling times. However, the addition of the *ae* allele to *wx* produced starch with an apparent amylose of 31% at 18 days post-pollination. In contrast to the typical increase in amylose percentage during kernel development (6,9), *ae wx* amylose percentage declined (Fig. 1). Thus, in *normal, ae,* and *ae su* the rate of amylose accumulation was greater than that of amylopectin with increasing kernel age, but the reverse was true for *ae wx.*

Consideration of the changes in relative activity of various enzymes during endosperm cellular development is useful in interpreting these patterns of amylose accumulation (Fig. 1). The developing maize kernel is composed of cells of varying physiological ages (1,2,3) and cells in the earliest stages of starch biosynthesis may produce starch from UDP-glucose by the action of granule-bound UDP-glucose starch glucosyltransferase (3,5). Later in development the activity of soluble ADP-glucose-starch glucosyltransferase and phosphorylase II and III increases (5,17). Schiefer *et al.* (18) suggested that amylose is produced *in vivo* by the action of a soluble ADP-glucose-starch glucosyltransferase while amylopectin is produced by an enzyme complex of ADP-glucose-starch glycosyltransferase and branching enzyme. Also, based on a polyacrylamide gel electrophoresis study, they (18) showed that *ae* kernels contained higher activity in the free glucosyltransferase bands than did *normal.* Another theory for *ae* action given by Shannon and Creech (19) suggests that *ae* may contain a debranching enzyme which would remove branches from amylopectin yielding a higher proportion of amylose and low-molecular-weight linear polysaccharides. Regardless of the specific enzymes involved, the amylose percentage in the starch is the result of the relative activity of two or more enzymes or enzyme complexes. Thus, during cellular development it is likely that a change occurs in the relative activity of these enzymes resulting in the increased percentage of amylose in *ae-*

Ref, ae-il, ae su, and *normal,* and the decline in the percentage of apparent amylose in *ae wx* (Fig. 1).

Although others (10,20) have reported a near normal apparent amylose content in *ae wx* starch, Tsai (21) recently reported that unpublished data from his laboratory indicated that *ae wx* starch was exclusively amylopectin, but the maximum absorption of the starch-iodine complex was 580 nm as compared to 540 nm for *wx* starch. An amylopectin with an absorption maximum at 580 nm could have contributed to the apparent amylose estimated by the blue value procedure (13) used in the present study. If *ae wx* starch is composed only of amylopectin, then with increasing age either the average chain lengths decreased or the polysaccharides produced by physiologically older cells are more tightly branched. Additional study will be necessary to determine if *ae wx* starch contains only a modified amylopectin or a mixture of amylose and amylopectin.

The average granule sizes for *normal* and the various mutant starches (as determined by the Coulter Counter) varied from a minimum of 2.6 μ in diameter for *ae su* at 18 days post-pollination to 7.7 μ for *normal* at 36 days post-pollination (Table I). The average granule sizes were smaller than those reported by Wolf *et al.* (22) for *normal* and *ae* starches from mature kernels. This is not surprising since their samples were from mature kernels isolated by a commercial procedure which did not quantitatively recover the small granules. Mercier *et al.* (9) showed an increase in granule size with increasing kernel age. The average granule sizes of their *normal* and *ae* starch preparations were also larger than ours. This was probably because the starches for their study (9) were taken from the floury portion of kernels which contain the more mature cells (3). Brown *et al.* (23) determined the minimum and maximum granule dimensions of starches isolated from kernels 12, 18, and 24 days post-pollination. Average starch granule sizes, based on the measurement of 120 granules, were also larger than those we obtained with the Coulter Counter. These differences may have been due to the small sample size used by Brown *et al.* (23). Alternatively, our samples could possibly contain small nonstarch cellular constituents not observed when the starch preparations were microscopically checked for purity. Such an inclusion would inflate the number of small (1.5 to 2.5 μ) granules and result in a smaller overall average diameter. Although the average granule diameter given in this study may be smaller than previously reported (9,23), the great heterogeneity of granule size emphasizes the importance of changes in granule size distribution with increasing age.

The starch granule size distributions for *normal* and the various mutant types at 18, 24, and 36 days post-pollination are shown in Fig. 2. Actual numbers of granules counted to generate the distribution are given in Table I. Size distributions of *normal* starch granules at 18, 24, and 36 days after pollination indicated that the greatest change in granule size occurred between 18 and 24 days (Fig. 2a). However, at all times after pollination, significant numbers of the granules were less than 5 μ in diameter. Size distributions of starch granules from all mutant genotypes showed a general reduction in maximum granule size, and a repression of granule enlargement compared to *normal*, but those from *wx* most closely resembled *normal* (Fig. 2b through 2f). The *ae* genotypes produced smaller starch granules but the maximum granule size was larger in *ae-il* than in *ae-Ref* starch (Fig. 2c and 2d). Size distributions from *ae wx* (Fig. 2e) showed greater reduction in granule dimensions than either *ae-Ref* or *ae-il*. Furthermore,

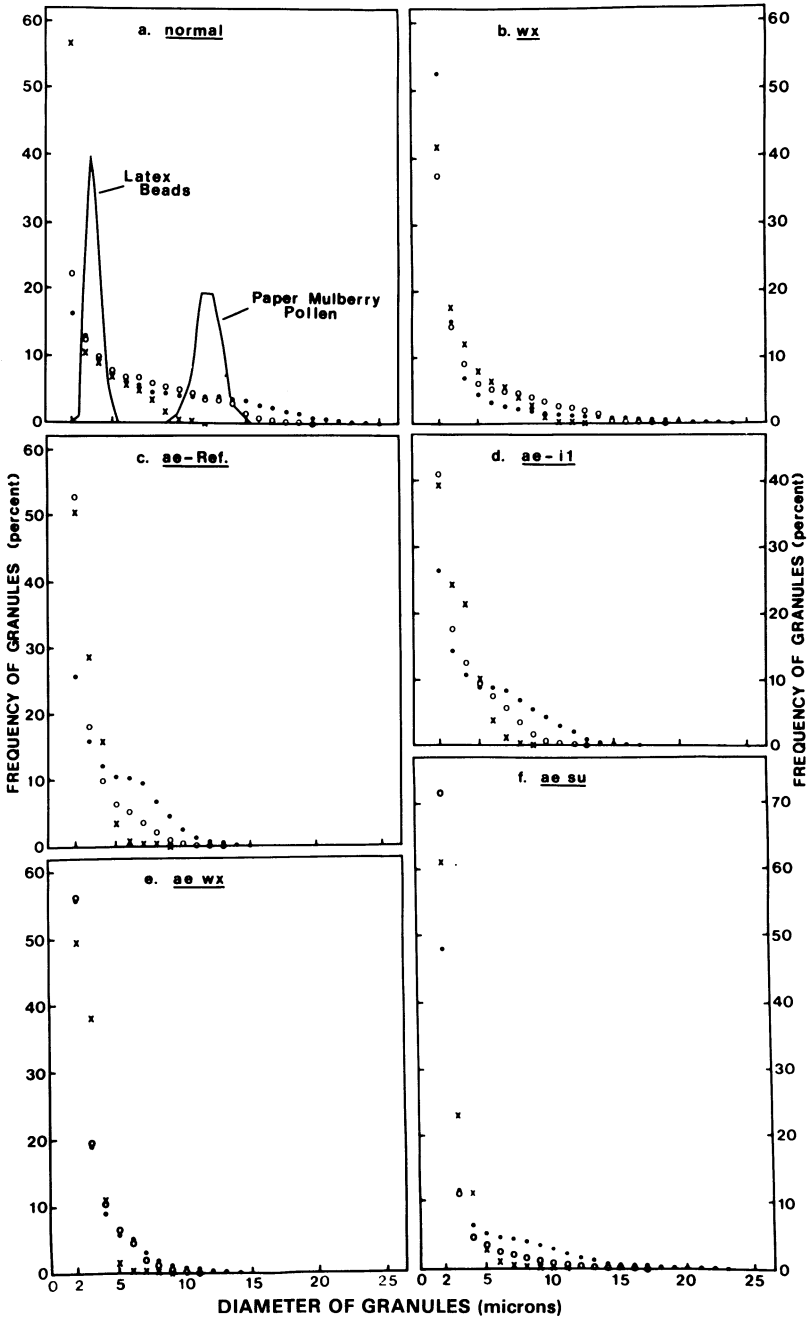

Fig. 2. Size distribution of starch granules of six maize genotypes at 18 (x's), 24 (open circles), and 36 (solid circles) days post-pollination. Solid lines in Fig. 2a represent size distributions for latex bead and paper mulberry pollen standards used for calibration.

very little granule enlargement occurred after 24 days post-pollination. Starch from *ae su* differed from all others in that at all ages a large proportion of granules were less than 5 μ in diameter but a few starch granules were almost as large as the largest granules from *normal*. In contrast to *ae wx*, considerable increase was observed between 24 and 36 days post-pollination in the number of *ae su* granules larger than 5 μ.

Possibly, starch granules isolated late in kernel development from physiologically younger cells may reflect the characteristics of starch isolated from the whole endosperm early in kernel development. Gradients of cellular development within the maize kernel have been confirmed by histochemical analyses (1) and by analysis of endosperm zones (4). Further evidence for differences in starch granules has come from reduced incorporation of ^{14}C into starch granules per unit surface area for starch granules containing less than 0.1 γ of starch (3). To test whether smaller starch granules have different amylose percentages when compared to unfractionated starch or larger granules from the same sample, and thus reflect the developmental patterns for amylose accumulations, granules were fractionated by size and apparent amylose percentage measured. Figures 3 and 4 show examples of granules of the size classes separated by dry microsieving and settling through glycerol, respectively.

Smaller starch granules separated from starch of 36-day-old *normal*, *ae-Ref*, and *ae su* kernels might be expected to show a lower amylose percentage than the unfractionated starch, because in all three genotypes, the amylose content of the starches increased with kernel development (Fig. 1). This pattern was observed for the smaller starch granules in all three genotypes (Table II). Starch granules below 5 μ in all three genotypes had amylose percentages near that of unfractionated starch isolated from kernels harvested 24 days post-pollination. Further reduction of starch granule size of *ae-Ref* and *ae su* starch reduced the amylose percentage, but a percentage near that of 18-day starch was not obtained (Table II). However, these starch fractions contained a large proportion of abnormally shaped granules (Fig. 4). The number of abnormal granules increases with development (9,24) and could result from an increasing number of

TABLE I
Number and Average Size of Granules Counted for Starch Granule Size Distributions given in Fig. 2a through 2f

Genotype	Days Post-Pollination					
	18		24		36	
	Number	Diameter[a] μ	Number	Diameter μ	Number	Diameter μ
normal	161,494	3.3	83,671	5.8	80,336	7.7
wx	120,235	3.9	136,792	4.9	256,147	4.1
ae-Ref	53,443	2.8	234,616	3.2	120,555	4.7
ae-il	56,571	3.2	203,244	3.7	169,363	5.1
ae wx	152,642	2.7	313,727	2.9	365,583	3.1
ae su	129,251	2.6	559,722	2.8	182,393	4.5

[a]Average diameter of all particles counted to generate the size distributions. Granule diameter is calculated from the volume measured by the Coulter Counter, assuming that the granules are spheres.

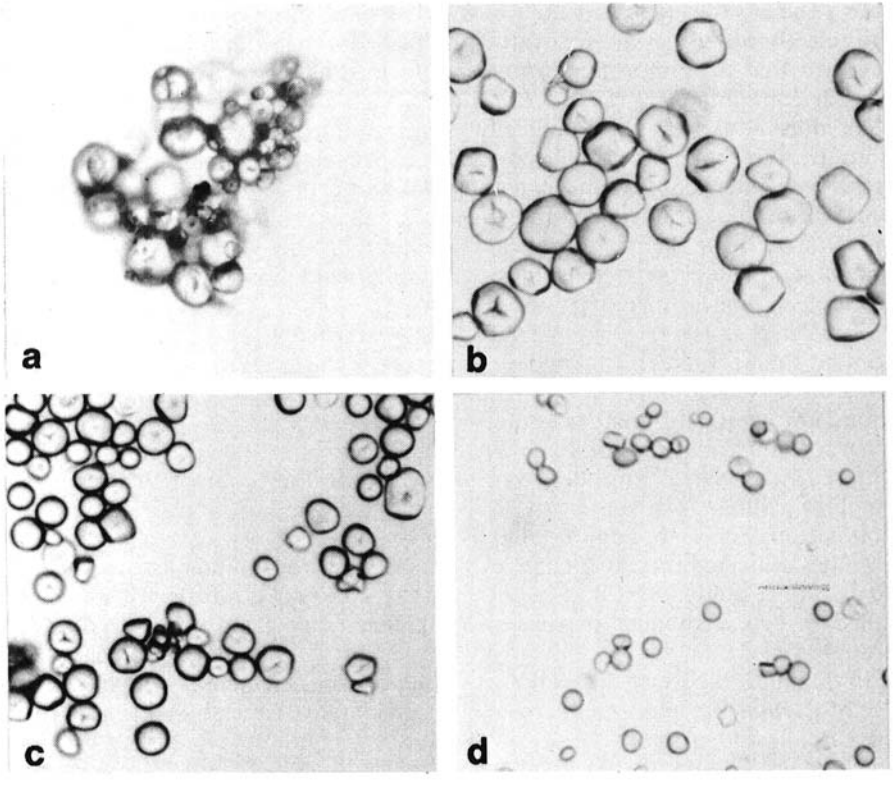

Fig. 3. Examples of *normal* starch granules of different sizes separated by dry microsieving. a) granule clump greater than 20 μ, b) 10 to 20 μ, c) 5 to 10 μ, and d) less than 5 μ. Magnification 256×.

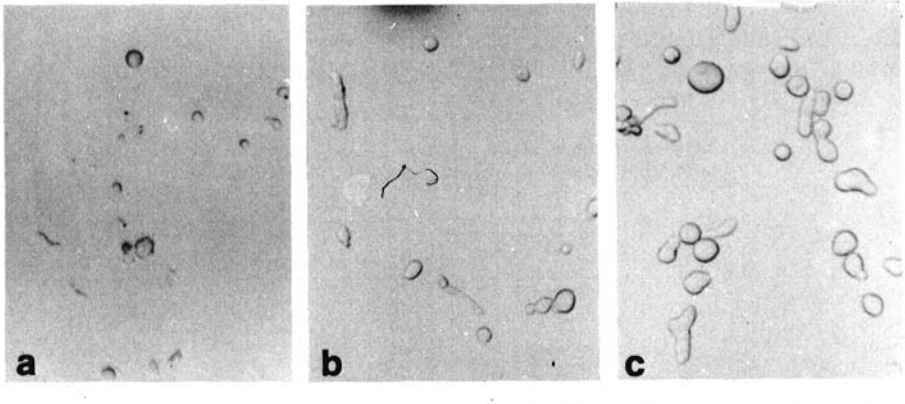

Fig. 4. Examples of small *ae-Ref* starch granules of different sizes separated by settling through 30% glycerol. a) Fraction C-1, b) fraction C-2, and c) fraction C-3. Magnification 256×.

cells reaching the stage of starch biosynthesis and of *ae* expression when these granule abnormalities are formed. Unpublished observations in our laboratory revealed that the abnormal granules are formed as secondary granules in cells already containing large spherical granules. Since these granules are produced in cells more advanced physiologically than those producing the small spherical granules, the abnormal granules would be expected to have the amylose content reflective of the genotype at a later stage of kernel development. Therefore, the presence of these abnormal granules in the small granule fractions of *ae-Ref* and *ae su* may have kept the amylose content of these starches higher than anticipated. We propose that the smaller spherical granules are indeed derived from physiologically younger cells.

Smaller granules of *ae wx* starch would be expected to be higher in amylose content than the unfractionated starch because the amylose content decreased with kernel development (Fig. 1). No differences in amylose percentages were found among the different size fractions of *ae wx* starch (Table II). Two factors may account for the inability to detect a difference in the amylose content of different sized starch granules from *ae wx* starch. First, only a 10% change in amylose content was observed in starches between 18 and 36 days post-pollination (Fig. 1). In addition, the size distributions generated for *ae wx* starch granules indicated very little change in the size of starch granules between 24 and 36 days post-pollination. Because of these two factors it is not surprising that we failed to detect amylose percentage differences associated with the different granule sizes.

In this study we showed that the amylose percentage increased with increasing age of *normal, ae,* and *ae su* kernels. This increase was due at least in part to an increase in the proportion of larger granules containing a higher amylose percentage. The small spherical starch granules which had lower amylose percentages presumedly were isolated from physiologically younger cells. Tsai *et al.* (5) suggested that there is a shift in starch biosynthetic enzymes in kernels as they begin active starch synthesis, and Shannon (3) proposed that such changes occur in each endosperm cell as it begins active starch synthesis. Therefore, the increased amylose percentage with age is a reflection of the advancing average physiological age of the total endosperm cell population. The single mutant effect and double mutant interactions appear to be expressed at different times in cellular development. Additional study will be necessary to determine what

TABLE II
Amylose Content of Starch Granules of Various Size Classes Isolated from Four Maize Genotypes 36 Days Post-Pollination

Size Class	Genotype			
	ae-Ref	*ae su*	*normal*	*ae wx*
	% of total starch[a]			
Unfractionated	67.8ab	59.0a	25.4a	22.6a
10 to 20 μ	69.5a	61.0a	26.4a	25.6a
5 to 10 μ	64.4b	53.6b	23.0ab	24.1a
Less than 5 μ	58.3c	47.0cd	20.5b	25.4a
C-3	63.8b	48.4c	...	...
C-2	57.3cd	43.6cd	...	...
C-1	53.0d	43.3d	...	...

[a]Values followed by the same letter within a column are not significantly different ($k = 100$).

enzyme changes are responsible for the mutant effects on starch composition.

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