Size-Distribution of Wheat Starch Granules During Endosperm Development

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ABSTRACT

Starch granule development in hard red winter wheat was followed by light and electron microscopy and quantitative image analysis. Proplastids in the cytoplasm of the coenocytic endosperm two days after flowering (DAF) differentiated into amyloplasts by 4 DAF. Quantitative image analysis showed that the starch granules initiated during the first 4 DAF increased in size to mean diameter of 5.6 μm by 7 DAF. These early synthesized granules (type A) continued to enlarge to their maximum diameter of 25–50 μm, some as soon as 19 DAF. A second group of starch granules, the small type B granules, was initiated at 10 DAF, was pronounced by 12 DAF, and did not enlarge during 12–19 DAF. These small granules began to enlarge at 21 DAF but only grew to a mean diameter of about 9 μm by maturity (35 DAF). A third group (designated type C) of small granules was initiated at 21 DAF. At maturity three distinct size groups of starch granules were present: large type A granules with diameters greater than 15.9 μm, type B granules with equivalent diameters between 5.3 and 15.9 μm, and small type C granules with equivalent diameters less than 5.3 μm. At maturity, the total number of starch granules comprised 45.7% type C granules, 49.5% type B, and 4.8% type A. The type C granules constituted 3.4%, type B 45.0%, and type A granules 51.6% of the total mass at maturity.

Starch is the major component of wheat endosperm, composing approximately 64–74% (14% moisture basis) of milled endosperm (Pomeranz 1988). Wheat starch is unique because it cannot be replaced by corn, rice, or oat starches or by noncereal starches to yield a satisfactory baked product (Hoseney et al 1971). The starchy endosperm of wheat at maturity is thought to contain two sizes of starch granules, the large type A and the smaller type B, similar to the ones found in barley (May and Buttrose 1959). A possibility exists of more than two size populations of granules in starches isolated from New Zealand wheats (Meredith 1981, Baruch et al 1983).

Starch, in the form of distinct granules, is synthesized in an organelle called the plastid. In endosperm tissue, a specialized type of plastid called the amyloplast is responsible for the synthesis and formation of the starch granules. Two distinct classes of granules based on size, large type A and small type B granules, are generally synthesized in separate plastids or within distinct compartments of the same plastid (Parker 1985, May and Buttrose 1959). The different sizes of granules have been identified using microsieve, Coulter Counter, and quantitative image analyses (Evers and Lindley 1977; Brocklehurst and Evers 1977; Baruch et al 1979, 1983; Karlsson et al 1983; Soulaka and Morrison 1985; Morrison and Scott 1986; Bechtel et al 1986, 1987) and showed a bimodal distribution.

Little information is available on the development of granules in wheat. It is generally thought that the large type A granules are synthesized first and the small type B ones later in development (Karlsson et al 1983). Most of the studies on granule size distribution used the Coulter Counter (Evers and Lindley 1977; Brocklehurst and Evers 1977; Baruch et al 1979, 1983; Karlsson et al 1983; Soulaka and Morrison 1985; Morrison and Scott 1986). This instrument analyzes large numbers of granules but does not accurately measure particles with diameters less than 3 μm (Karlsson et al 1983) unless changes are made in the orifice tubes. As a result, starch granules smaller than 3 μm are not typically counted in the particle size determination when the Coulter Counter is used.

Various reports have linked granule size to different rheological properties (Kulp 1973, Rasper and deMan 1980), baking characteristics (D'Appolonia and Gilles 1971), and compositional differences (Meredith 1981). It is therefore necessary to have an accurate method that measures all of the starch granules present in a preparation.

The work we report here uses quantitative image analysis coupled with dark field microscopy of isolated starch granules to follow granule development in a hard red winter wheat. The results obtained suggest a trimodal distribution of starch, rather than a bimodal one, with the third class of granules being significant in number rather than in mass.

MATERIALS AND METHODS

Samples and Starch Isolation

Field-grown hard red winter wheat, cultivar Newton, was grown near Manhattan, KS, during the 1985 growing season. Heads were tagged at anthesis and harvested at 7, 10, 12, 14, 17, 19, 21, 24, 28, and 35 (maturity) days after flowering (DAF). Individual caryopses were either frozen at −70°C or used fresh. Caryopses were degermed by cutting the bottom one-third of the germ end off using a clean, new razor blade. The endosperm was then removed from the remainder of the caryopsis by gently squeezing the top portion of the grain. The endosperms were squeezed into 4°C buffer (25 m*M* Tricine, 5 m*M* magnesium acetate, 50 m*M* potassium acetate, pH 7.5). A two-to-one volume ratio of buffer to endosperms was used. The mixture was homogenized in a Tekmar Tissumizer (model SPT, Cincinnati, OH) with high-torque speed control (model TR-5T) at a 20–25 setting for 30–60 sec. The resulting slurry was squeezed through a single layer of sterile gauze into 1.5-ml microfuge tubes, rinsed with 0.5 ml of buffer, and spun for 20 sec in an Eppendorf 5412 centrifuge. The supernatant was discarded, and the pellet was resuspended in water and recentrifuged. This washing process was conducted two or three times depending upon stage of development and degree of contamination of cellular debris, which formed a layer at the starch-buffer interface. Care was taken that tailings starch at this interface was not lost. Starch yields were calculated on a dry weight basis from lyophilized starch obtained from a known number of caryopses and an estimated moisture content of the caryopses (Table I). The dry weight of these caryopses was determined by weighing a number of lyophilized caryopses not used in the isolations. Two losses of starch occurred: one when the germ end was removed, and the other when the cell debris was filtered through the gauze. Neither is thought to influence the distribution of starch profiles.

Microscopy

The purified starch was checked using bright field microscopy with crossed polarizers to check for contamination and for starch damage. These microscopic analyses revealed no visible damage to starch due to isolation procedures and revealed no visible

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contamination of the starch with nonstarch particles of similar size. Samples of starch were lyophilized and stored at −20°C until used.

Analysis of starch was conducted by rehydrating a portion of the starch in water and mixing it with glycerol to make about a 10% solution (to cut down on Brownian movement). The starch slurry was thoroughly mixed, a small drop placed on a microscopic slide, and a cover glass placed over the suspension. Isolated starch was studied with a variety of light microscopic techniques, including bright field, phase contrast, polarized light, and darkfield microscopy, to determine which one was best suited for image analysis in an unstained state. Darkfield microscopy gave the highest contrast and sharpest images of all the light microscopic techniques and was most conducive for use in the quantitative image analysis system. Therefore, darkfield microscopy was used throughout this study for image analysis. Random fields were photographed and the negatives printed on high-contrast paper. Starch granules that were touching one another were "manually" separated with black ink, and dark centers within the starch granules were whitened out using typing correction fluid, because the image analysis system lacked an image edit mode. These hard copies of the slides were then used for the quantitative image analysis. Starch in 4 DAF caryopses was found mainly in the pericarp; there was little in the starchy endosperm. Starch in the 4 DAF caryopses was analyzed using sectioned material as previously described (Bechtel et al 1982), and the starch was measured directly from transmission electron micrographs of the sections. Scanning electron microscopy (SEM) was conducted as previously described (Pomeranz et al 1977) and was used to determine the thickness of starch granules at each stage of development by directly measuring the starch granules in the electron micrographs.

Quantitative digital image analysis was used to evaluate number, shape, and size of isolated starch granules. Micrographs of the isolated starch were analyzed by a Cambridge (Imanco) Quantimet 720 image analyzer upgraded to model 23A (see description of the system, Zayas et al 1986) coupled to a Digital Equipment Corporation PDP 11/03 microcomputer with 32-kB memory. A Vidicon camera with a Nikon 55 mm/f 2.8 lens was used as an input device for image acquisition and had a standard frame of 800 × 625 pixels that incorporated a guard area to eliminate edge distortions. Video images were calibrated in micrometers. Shade correction was performed before image digitization to avoid effects of nonuniform illumination. A photograph with the same ratio of white to black field as sample photographs was used to calibrate the system. Digitized six-bit gray value images (64 gray levels) were stored and analyzed either on- or off-line. Acquired parameters, such as starch granule number, area, and perimeter were used to calculate equivalent diameter and circularity shape factor. The number of fields measured varied from 12 to 24 depending on the number of starch granules in the field of view. The number of granules evaluated for this study ranged from about 300 to 800. Equivalent diameter, the diameter of a circle with an area equal to that of the measured starch granule, was calculated by the following formula:

TABLE I
Yield of Starch From Wheat Caryopses

Stage of Development (DAF)[a]	Starch Yield (% dry weight)[b]
7	3.0 ± 1.0
10	6.6 ± 1.6
14	14.3 ± 0.7
17	23.5 ± 2.5
21	32.3 ± 0.7
24	34.3 ± 0.3
28	34.3 ± 0.3
35	36.0 ± 0.7

[a] Days after flowering.
[b] Dry weight basis of entire caryopsis.

$$\text{Equivalent diameter} = [(\text{measured feature area} / \pi)^{0.5}] \times 2$$

The circularity shape factor, the relationship comparing the shape of a starch granule with that of an ideal circle, was calculated from the following equation:

$$\text{Circularity shape factor} = \text{feature perimeter}^2 / 4\pi \times \text{feature area}$$

Surface area and mass calculations were based on the equivalent diameter, specific gravity of starch of 1.5×10^{-12} g/μm^3, and the assumption that granules less than 5 μm in diameter were spherical and those greater than 5 μm were disks. Thickness of the starch granules was measured directly from scanning electron micrographs. Each set of data was normalized to a standard relative number to eliminate variations due to the number of granules measured. The following formulas were used in the calculations:

$$\text{Surface area of a sphere} = (\text{equivalent diameter}^2/2) \times \text{normalization factor} \times 4\pi$$

$$\text{Surface area of a disk} = (\text{equiv. diam.} \times \pi T) + (\text{equiv. diam.}^2/2)\, 2\pi \times \text{norm. factor}$$

$$\text{Mass of a sphere} = (\text{equiv. diam.}^3/2) \times \text{norm. factor} \times 4/3\pi \times 1.5 \times 10^{-12}$$

$$\text{Mass of a disk} = (\text{equiv. diam.}^2)\, \pi T \times \text{norm. factor} \times 1.5 \times 10^{-12}$$

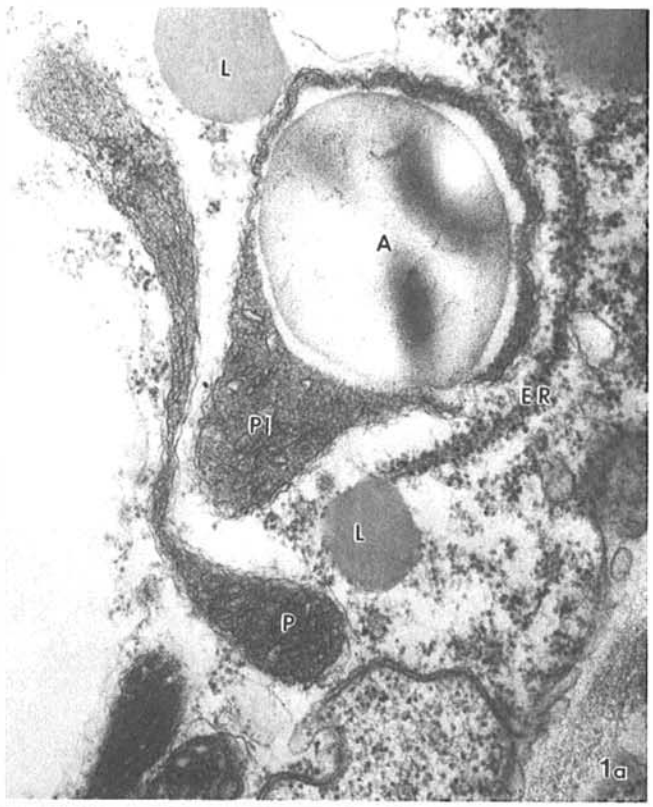
where T = thickness of starch granule at that particular stage of development, and the specific gravity of starch is 1.5×10^{-12} g/μm^3.

RESULTS

Starch granules were found within amyloplasts with only one granule present in each plastid (Fig. 1). Analysis of plastic thin sections of 4 DAF endosperm viewed with transmission electron microscopy revealed a single population (destined to become the large type A granules) of starch with a mean diameter of about 1.8 μm (Figs. 1 and 3a). Darkfield microscopy gave the highest contrast, sharpest edges, and best resolution of the various microscopic techniques tried and was used exclusively for the image analysis of samples of starch isolated from 7 to 35 DAF (Fig. 2). Quantitative image analysis of isolated 7 DAF starch exhibited a single population of granules (enlarging type A granules) with an average diameter of 7 μm and a maximum diameter of 12 μm (Fig. 3b). The 10 DAF isolated starch showed a broad band of variously sized granules up to 30 μm diameter (Fig. 3c). By 12 DAF, however, a burst of synthesis created a new population of small starch granules (type B granules) that averaged 1.7 μm in diameter (Fig. 3d). This general profile of starch granule distribution was maintained (Fig. 3d–g) up to 21 DAF, at which time a broader band of granules with an average diameter of about 5 μm was observed (Fig. 3h). By 24 DAF, however, a new distinct class of small granules, which we call type C granules, was synthesized (Fig. 3i). The 24 and 28 DAF starch showed three distinct populations of granules (>16 μm, type A; 5–16, type B; 0–5, type C) with peaks at 2 μm, 9 μm, and a broad band (small percentage of the total) of granules larger than 10 μm (Fig. 3i and j). The three population characteristics persisted at maturity (Fig. 3k).

Circularity shape factor (CSF) is the relationship between the shape of the measured object and an ideal circle. A perfect circle would have a CSF of one. Any deviation from one indicates that the object is not a circle. Starch granules used in this study had CSF values ranging from 1.08 to 1.12 indicating that the starch was nearly circular (Table II). Starch granules, particularly those of type A, are known to be "lens-shaped" and would be most likely to lie on the major axis rather than present an edge view. Most views of starch from sectioned material reveal granules that are somewhat oblong as a result of sectioning at an angle

with respect to the major axis. SEM was used to follow granule thickness, and the data are summarized in Table II. Granules did not thicken appreciably until 21 DAF and then rapidly increased in thickness to a maximum of 5.0 μm (Table II).



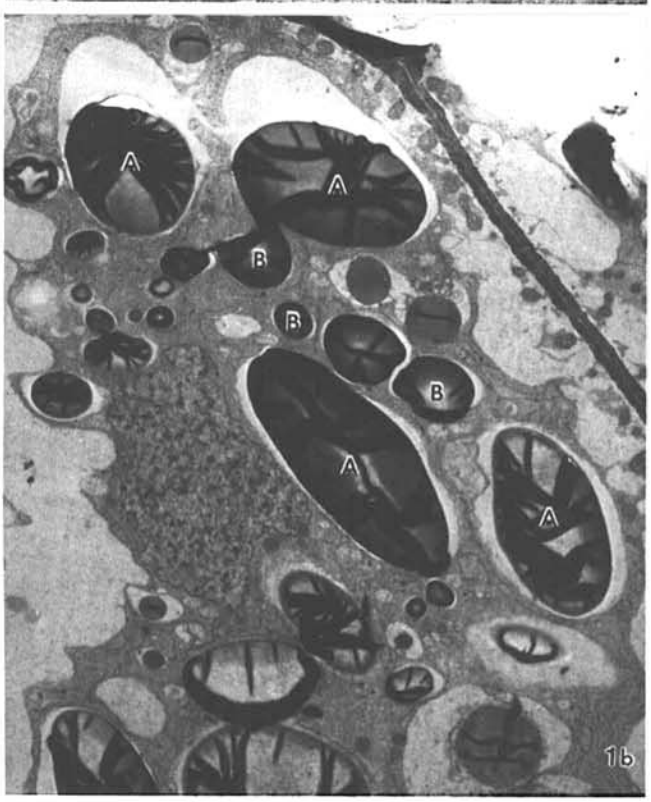


Fig. 1. Transmission electron micrograph of wheat starchy endosperm showing starch granules in amyloplasts. **A,** Endosperm from wheat seven days after flowering showing single incipient type A granule (**A**) in plastid (**PI**) next to a proplastid (**P**) that has not yet differentiated into an amyloplast: **L** = lipid droplet, **ER** = endoplasmic reticulum (×42,080). **B,** Type A and type B granules in endosperm 17 days after flowering (×3,280).

Equivalent diameter and thickness of starch were used to calculate the volume and mass of each group of granules. Volume of starch granules was computed assuming that granules less than 5 μm in diameter were spheres and those larger than 5 μm were disks of *T* thickness as measured from SEM micrographs (Table II). The percent surface area of each starch granule class was calculated for the 11 stages of development (Table III). The type B granules occupied 59.5% of the total surface area of 35 DAF starch, with only 33% composed of the large type A granules. The amount of surface area occupied by the type A granules generally decreases as maturity approaches (a drop from a high of 77.8% at 17 DAF to 33.0% at 35 DAF). Fluctuations in the amount of surface area for each of the starch size classes were substantial (Table III), reflecting the patterns of synthesis of the three granule classes.

The percentage of mass for each starch class was also calculated and revealed that more than half of the total mass at maturity (35 DAF, 51.6%) was in the large type A granule. If the calculations were based on starch granules being spheres rather than disks, the 35 DAF starch mass would be distributed as follows: 0–5 μm 1.2%; 5–16 μm 14.2%; >16 μm 84.6%. The total amount of mass exhibited by the very small granules was only 3.4% of the total (Table III), but because the surface area of a sphere increases by the square of the radius in comparison to the volume (mass), which increases by the cube, these small granules exhibit a substantial amount of surface area (7.5%) of the total at maturity (Table III).

DISCUSSION

The data reveal three classes of starch granules present in the mature wheat endosperm analyzed in this study. Based upon size distributions of starch isolated from developing endosperm, the classes are distinct. The the timing of initiation of each granule class provides further evidence that the classes are distinct. Type A granules form first (Buttrose 1963, Parker 1985) during the first few days of endosperm formation. At approximately 10 DAF,

Fig. 2. Light micrograph of isolated starch 24 days after flowering photographed with darkfield microscopy showing high contrast useful for image analysis (×112.5).

TABLE II
Circularity Shape Factor and Thickness of Isolated Wheat Starch

Stage of Development (DAF)[a]	Circularity Shape Factor	Thickness[b] (μm)
7	1.09	1.8
10	1.09	1.8
12	1.11	1.8
14	1.12	2.0
17	1.09	2.0
19	1.11	2.0
21	1.10	2.5
24	1.08	2.5
28	1.10	5.0
35	1.09	5.0

[a] Days after flowering.
[b] Measured from scanning electron micrographs.

another burst of starch granule synthesis begins and the type B granules are initiated; the interesting feature of these granules is their apparent lack of enlargement during the following 10 days. This could be due in part to the growth in diameter of the type A granules during this time (Fig. 3a–g). The observation of a third burst of granule synthesis (type C granules) at 21 DAF also lends credence to the fact that the groups of starch are distinct. What is not known at present is whether new granules would be synthesized at 10-day intervals if the growing season were extended beyond 35 DAF.

Why three distinct groups of granules? The main reason for identifying three granule classes now lies in the isolation procedure and method of measurement. Our isolation procedure allowed for excellent recovery of the small granules, which otherwise might be lost and not counted (Evers 1973). Secondly, the image analysis procedure we employed counted all starch granules that could be photographically identified. The Coulter Counter cannot count

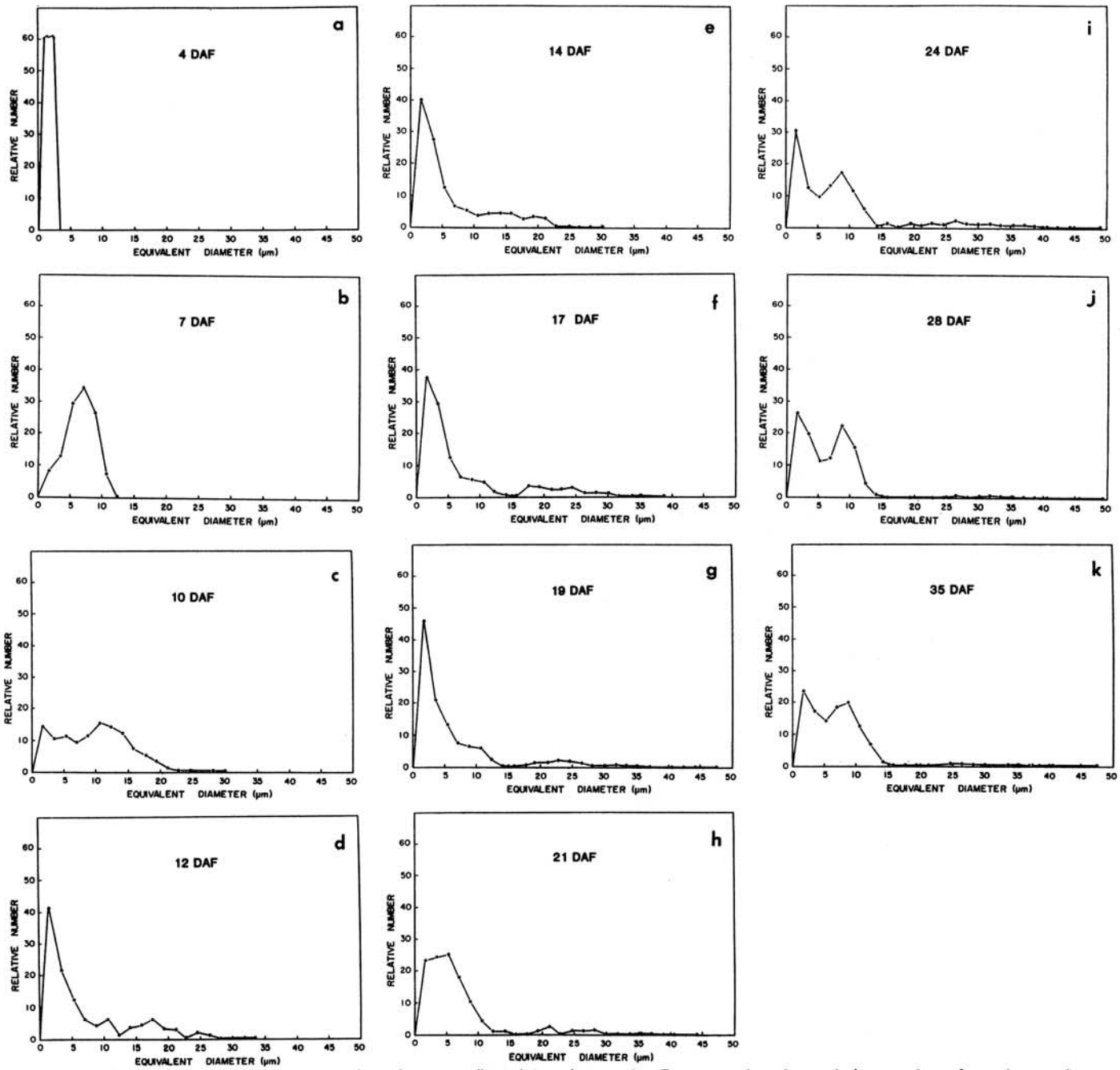


Fig. 3. Quantitative image analysis data obtained from darkfield light micrographs. Data are plotted as relative number of starch granules vs. equivalent diameter at various days after flowering (DAF; 35 DAF = maturity).

TABLE III
Percentage of Starch Granules in Three Size Classes

Stage (DAF)[a]	0-5 μm	5-16 μm	>16 μm
Based on surface area			
4	100	0	0
7	17.4	82.6	0
10	5.1	64.2	30.7
12	9.2	26.9	63.9
14	13.3	43.3	43.4
17	8.8	13.4	77.8
19	9.3	19.7	71.0
21	13.1	25.7	61.2
24	3.7	24.7	71.6
28	6.6	53.6	39.7
35	7.5	59.5	33.0
Based on mass			
4	100.0	0	0
7	10.6	89.4	0
10	5.4	61.0	33.6
12	8.5	24.8	66.7
14	11.6	42.0	46.4
17	6.8	15.1	78.1
19	7.5	17.5	75.0
21	10.5	21.1	68.4
24	25.	19.6	77.9
28	2.9	44.2	52.9
35	3.4	45.0	51.6

[a] Days after flowering.

granules much smaller than 3 μm (unless different orifice tubes are used), because this starch is below the resolution limits of the instrument (Karlsson et al 1983). As isolation techniques continue to improve, the number of small granules recovered will also increase. When the starch removed with the germ is also recovered the percent recovery will also increase. Although the number of small granules is significant, the mass they contribute to the total population is quite small (Table III). The small granules (tailings starch) affect water absorption (Hoseney et al 1971), possibly because of their high surface-to-volume ratios.

Our data depict a cutoff around 16 μm for differentiating between type A and B granules rather than the more commonly cited values of 10–12 μm (Parker 1985, Evers and Lindley 1977, Brocklehurst and Evers 1977, Morrison and Scott 1986, Karlsson et al 1983). This difference may relate to the method of measurement and standardization. The Coulter Counter is standardized using latex spheres of known volume. Starch granules are not spherical, however. Calculations based on starch being spherical lead to smaller values for diameters than those based on disks, particularly for starch granules with large volumes. The major advantage of the Coulter Counter is the large number of starch granules that can be analyzed, whereas two disadvantages are the loss of granules less than 3 μm in diameter and the smaller diameters calculated from the volumes. Quantitative image analysis allows measurement of all the granules that can be photographed, but relatively few granules can be studied. Future studies using an automated system should allow for many more granules to be analyzed.

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