

Endosperm Structural and Biochemical Differences Between a High-Protein Amphiploid Wheat and its Progenitors¹

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ABSTRACT

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A new high-protein amphiploid wheat was compared to its diploid (*Triticum tauschii* biotype *strangulata*) and tetraploid (*T. turgidum* var. *dicoccoides*) progenitors and to a commercially grown hexaploid hard red winter wheat (*T. aestivum* cv. Newton) all grown under the same conditions. The comparisons included light and transmission electron microscopy,

quantitative image analysis, and polyacrylamide gel electrophoresis of extracted prolamines. The amphiploid was found to possess endosperm characteristics of both the progenitors as revealed by various microscopic techniques. Polyacrylamide gel electrophoresis of gliadins showed that the amphiploid contained the same bands as both its progenitors.

The increase of bread wheat protein content, protein quality, and grain yield and improvement of other agronomic characteristics has been a long-time goal of wheat-breeding programs. Two factors have limited this goal: 1) the genetic information found in today's cultivated wheat has been fully exploited for breeding purposes, and 2) the range of genetic variation in cultivated wheats has declined during the past few decades of breeding (Feldman and Sears 1981). According to Feldman and Sears (1981), hexaploid wheat (genome AABBDD) originated as a hybrid between *Triticum turgidum* (probably the cultivated variety *T. turgidum dicoccum* or emmer wheat, genome AABB) and *T. tauschii* (genome DD). Spontaneous hybridization between *T. turgidum* and *T. tauschii*, the two progenitors of hexaploid wheat *T. aestivum*, probably took place in the field in western Iran about 8,000 years ago (Feldman and Sears 1981). Therefore, wild relatives of wheat may provide an untapped source of genetic variation useful in breeding programs. The use of techniques to introduce entire genomes of wild relatives of wheat into cultivated wheat led to the development of an amphiploid that crossed readily with hexaploid bread wheats (Worstell 1982). We undertook a structural and biochemical study of this amphiploid and its progenitors to determine which, if any, features were incorporated into the new synthetic wheat.

MATERIALS AND METHODS

Wheat Samples

Four wheat samples grown under identical greenhouse conditions were provided by James V. Worstell (University of Missouri, Columbia, MO). The diploid wheat, *T. tauschii* (Coss.) Schmal. biotype *strangulata* (*Aegilops squarrosa* L., *T. aegilops* P. Beauv. ex R. + S.) has the D genome where $2n = 14$. The tetraploid wheat, *T. turgidum* L. var. *dicoccoides* (Korn. in litt. in Schweinf.) Bowden (*T. dicoccoides* Korn. in litt. in Schweinf.) has the A and B genomes where $2n = 28$. Hexaploid bread wheat *T. aestivum* L. cv. Newton has the A, B, and D genomes where $2n = 42$. Worstell (1982) created an amphiploid by crossing the diploid and tetraploid wheats, then cultured tissue of excised 12-21 day old embryos. Seedlings regenerated from the tissue culture callus were treated with colchicine to double the chromosome number. One of these seedlings was an amphiploid wheat that had 21 chromosomes at meiosis and was used in this study.

Microscopy and Quantitative Image Analysis

Samples for transmission electron microscopy were prepared as described in Bechtel and Gaines (1982). Light microscopy and Coomassie Brilliant Blue protein staining were conducted using the methods of Bechtel and Pomeranz (1978). Image analysis was conducted on light micrographs of stained sections printed to very high contrast. A Quantimet 900 image analyzer (Cambridge Instruments) was used to measure the unstained and stained areas as a percentage of the total field. Between four and six micrographs each of the subaleurone and central starchy endosperm were analyzed. The area of protein measured cannot be used to quantify the actual amount of protein nitrogen present, because the dense stain masks nonprotein components (carbohydrate, lipid, inorganic compounds), and because the densities of starch and proteins are not the same. Similarly the starch fraction (nonstained region) includes small holes in the sections as well as cell walls. The data

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obtained, however, are quantitative in respect to the quantity of stained or unstained material.

Analytical Methods

Protein content was determined by AACC method 46-11 (1962). Average kernel weight was obtained by weighing the kernels in each sample and dividing the weight by the number of kernels. Polyacrylamide gel electrophoresis (PAGE) was conducted according to the methods of Lookhart et al (1982).

RESULTS

General

Various aspects of the amphiploid wheat and its progenitors are summarized in Table I. The amphiploid had a very high protein content (26.4%) and kernel weight (40.6 mg). The tetraploid had the highest protein content (28.7%) and next to highest kernel weight (35.7 mg). Both the diploid and hexaploid had much lower protein contents and kernel weights than the other two wheats.

The general appearance of the kernels differed substantially. Newton had the typical appearance of a hard red winter wheat (Fig. 1). The amphiploid wheat kernels were longer and darker in color than the Newton kernels (Fig. 2). The cross-sectional shape was triangular with a deep crease, but the cheeks were not as full as in Newton. In addition, the surface of the amphiploid kernels had numerous small shrunken areas. The tetraploid, *T. turgidum* had very long and narrow dark kernels that had a pronounced triangular cross section (Fig. 3). The cheek edges of these kernels were sharp rather than rounded, and the kernels had large, indistinct, shrunken regions. *T. tauschii* kernels were short, plump, and light in color (Fig. 4).

Light Microscopy

Aleurone cells of Newton, the amphiploid, and the tetraploid were all about the same size, whereas the diploid had much smaller aleurone cells (Figs. 5–8). A striking feature of Newton and the diploid was the thick cell walls of subaleurone cells (Figs. 5 and 8). By contrast, *T. turgidum* had very thin cell walls that were difficult to discern by light microscopy (Fig. 7). The amphiploid had cell walls similar to Newton and the diploid (Figs. 5, 6, 8). The subaleurone region was also the area of greatest difference in cell size and storage protein content. Subaleurone cells of *T. tauschii*

were small (Fig. 8), whereas cells of *T. turgidum* were both large and small (Fig. 7). Subaleurone cells of Newton and the amphiploid were mostly large (Figs. 5, 6).

Starch in the subaleurone cells also exhibited variation in size and distribution among the wheats. Newton had large (type A) and small (type B) starch granules surrounded by storage protein scattered throughout the cells (Fig. 5). The small type B starch granules of Newton were between 1 and 3 μm long, whereas the type A granules measured up to 13 μm long, with most in the 9–11 μm range. The amphiploid wheat had very large subaleurone cells with centrally located protein. The starch was primarily limited to the periphery of the cells and was composed mostly of groups of small starch granules and large granules that were between 5 and 7 μm long (Fig. 6). *T. turgidum* had mostly small type B granules in the subaleurone endosperm along with a few large (9–13 μm) type A granules. The large cells of the tetraploid had large masses of protein with peripheral starch (Fig. 7). *T. tauschii* had both large and small starch granules in all cells except the very small ones. These cells were too small to have the large type A granules in them. Storage protein was evenly distributed around the diploid wheat starch (Fig. 8).

Quantitative Image Analysis

Image analysis revealed several trends (Table II). The subaleurone region consistently had lower starch to protein ratios (higher protein content) than did the central endosperm. Cell size in the subaleurone region did not correlate with the amount of protein present in the cells. Newton, which had large cells, had the highest starch to protein ratio (1.69:1), indicating that the subaleurone region of Newton had more starch than protein. By contrast, *T. tauschii* had the smallest subaleurone cells and a starch protein ratio of only 1.17:1. Both the high-protein wheats (amphiploid and *T. turgidum*) had starch to protein ratios of less than one, indicating that there was more protein than starch in these subaleurone cells.

T. turgidum, which had the highest protein content, always showed the highest concentration of stained protein in both the subaleurone and central endosperm regions (62.0 and 36.3%). Newton had the lowest protein content and also showed the lowest amount of stained protein (37.4 and 24.2%). The other two wheats showed intermediate quantitative values (Table II). There was less variability in the subaleurone region (standard deviations ranged from 0.174 to 0.249) than in the central endosperm (standard deviations ranged from 0.327 to 1.03).

Electron Microscopy

Transmission electron microscopy was used to identify structural differences in the storage protein and cytoplasmic remnants of the wheats. The storage protein matrix in Newton was nearly homogeneous, with starch uniformly distributed (Fig. 9). Electron-dense spherical inclusions were dispersed throughout the matrix of some cells (Fig. 10). This distribution seemed to be found only in those oblique sections taken near the cell wall. Many cells have the dense inclusions arranged near the periphery of the thick cell wall. Most of the cytoplasmic remnants of Newton endosperm cells were small and widely dispersed around the cell perimeter (Fig. 9). Remnants consisted of ribosomes and rough endoplasmic reticulum (RER) in which the lumen contained medium electron-dense material (Fig. 11).

The amphiploid wheat had large areas of protein matrix in the central part of the cell (Fig. 12). The matrix was typified by numerous small inclusions, most being a flocculent electron-dense material (Fig. 13). Spherical dense inclusions were small (<1.5 μm in diameter) and scattered in the matrix (Fig. 13). Larger dense inclusions were limited to the cell periphery (Fig. 12). Some cells exhibited limited amounts of lamellar material in the storage protein (Fig. 13). The unique cytoplasmic remnants of the amphiploid were large and scattered throughout the cells. Many exhibited large amounts of ribosomes and RER (Figs. 14 and 15). In many areas the RER appeared to have ruptured and exuded large quantities of material into the protein matrix (Figs. 14 and 15).

TABLE I
Comparison of Amphiploid Wheat to its Progenitors

Wheat	Protein (%)	Avg. Kernel Wt. (mg)	Genome	2n Chromosomes
Newton	16.1	23.4	AABBDD	42 = 6 \times
Amphiploid	26.4	40.6	AABBDD?	42 = 6 \times
<i>T. turgidum</i>	28.7	35.7	AABB	28 = 4 \times
<i>T. tauschii</i>	16.7	13.1	DD	14 = 2 \times

TABLE II
Quantitative Image Analysis of Wheat Sections Stained with Coomassie Brilliant Blue

Wheat	Starch Fraction ^a	Protein Fraction ^b	Starch-Protein Ratio		Standard Deviation
			Average	Range	
Subaleurone Region					
Newton	62.6	37.4	1.69	1.41–2.00	0.246
Amphiploid	38.2	61.8	0.634	0.386–0.897	0.182
<i>T. turgidum</i>	38.0	62.0	0.628	0.396–0.871	0.249
<i>T. tauschii</i>	53.5	46.5	1.17	0.878–1.41	0.174
Central Endosperm					
Newton	75.8	24.2	3.17	2.74–3.80	0.411
Amphiploid	66.8	33.2	2.13	1.31–2.80	0.635
<i>T. turgidum</i>	63.7	36.3	1.78	1.47–2.28	0.327
<i>T. tauschii</i>	74.7	25.3	3.13	2.30–4.47	1.03

^a Measured as percentage of unstained field of view.

^b Measured as percentage of stained field of view. Nonprotein material in stained regions accounts for discrepancies in actual amounts of protein.

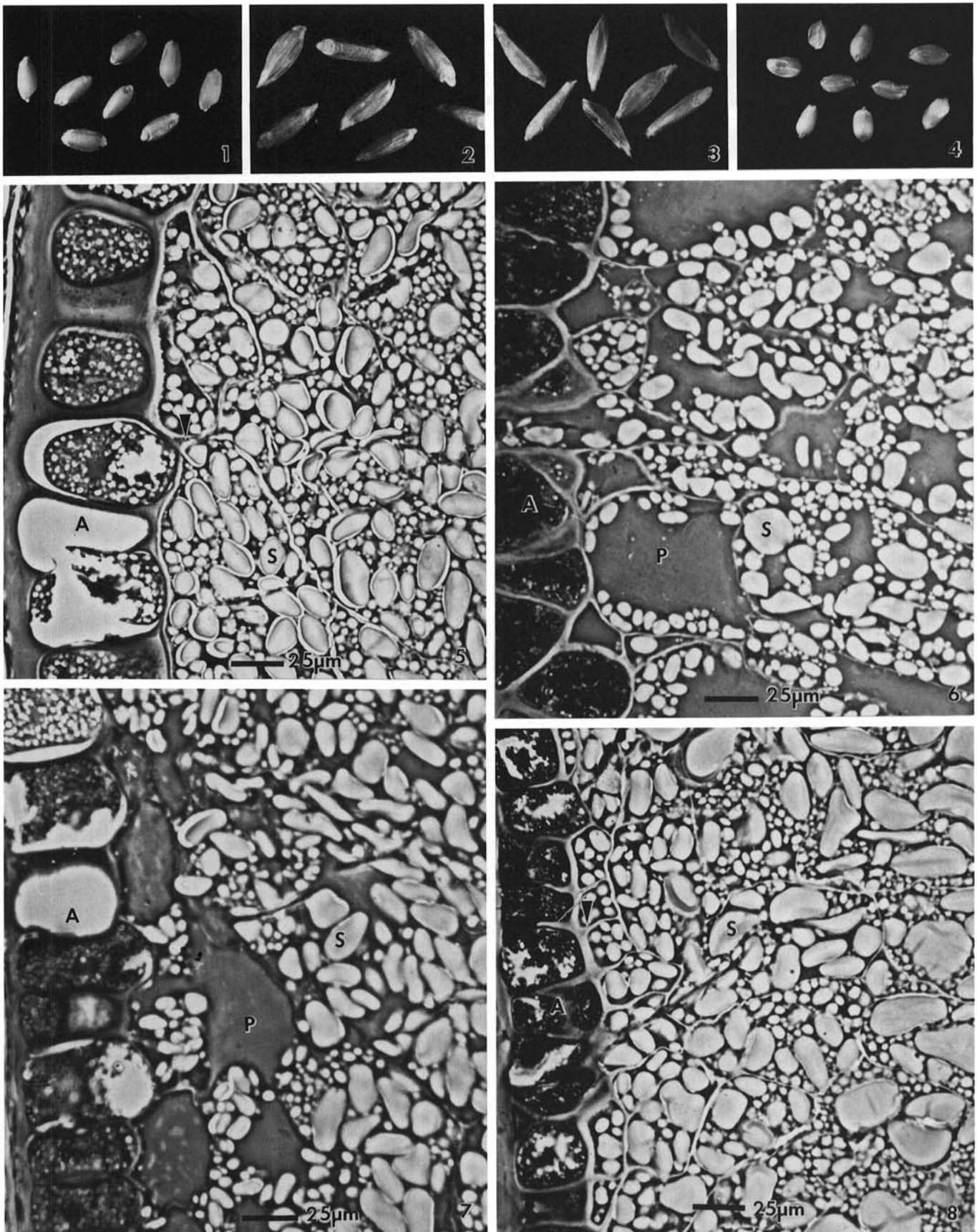


Fig. 1. View of hard red winter wheat Newton ($\times 1.3$). Fig. 2. Low magnification micrograph of the amphiploid wheat ($\times 1.3$). Fig. 3. View of tetraploid wheat *Triticum turgidum* ($\times 1.3$). Fig. 4. Primitive diploid wheat *T. tauschii* viewed at low magnification ($\times 1.3$). Fig. 5. Light micrograph of aleurone (A) and subaleurone endosperm of Newton showing starch granules (S) embedded in dense protein and the thick cell walls of the subaleurone cells (arrow) ($\times 400$). Fig. 6. Light micrograph of amphiploid aleurone (A) and subaleurone regions. Note large areas of storage protein (P) and limited number of starch granules (S) ($\times 400$). Fig. 7. Light micrograph of tetraploid, *T. turgidum* aleurone (A) and subaleurone regions. Storage protein (P) is in large areas and starch (S) is limited ($\times 400$). Fig. 8. Light micrograph of diploid, *T. tauschii* endosperm showing small aleurone cells (A), thick subaleurone cell walls (arrow), and numerous starch granules (S) ($\times 400$).

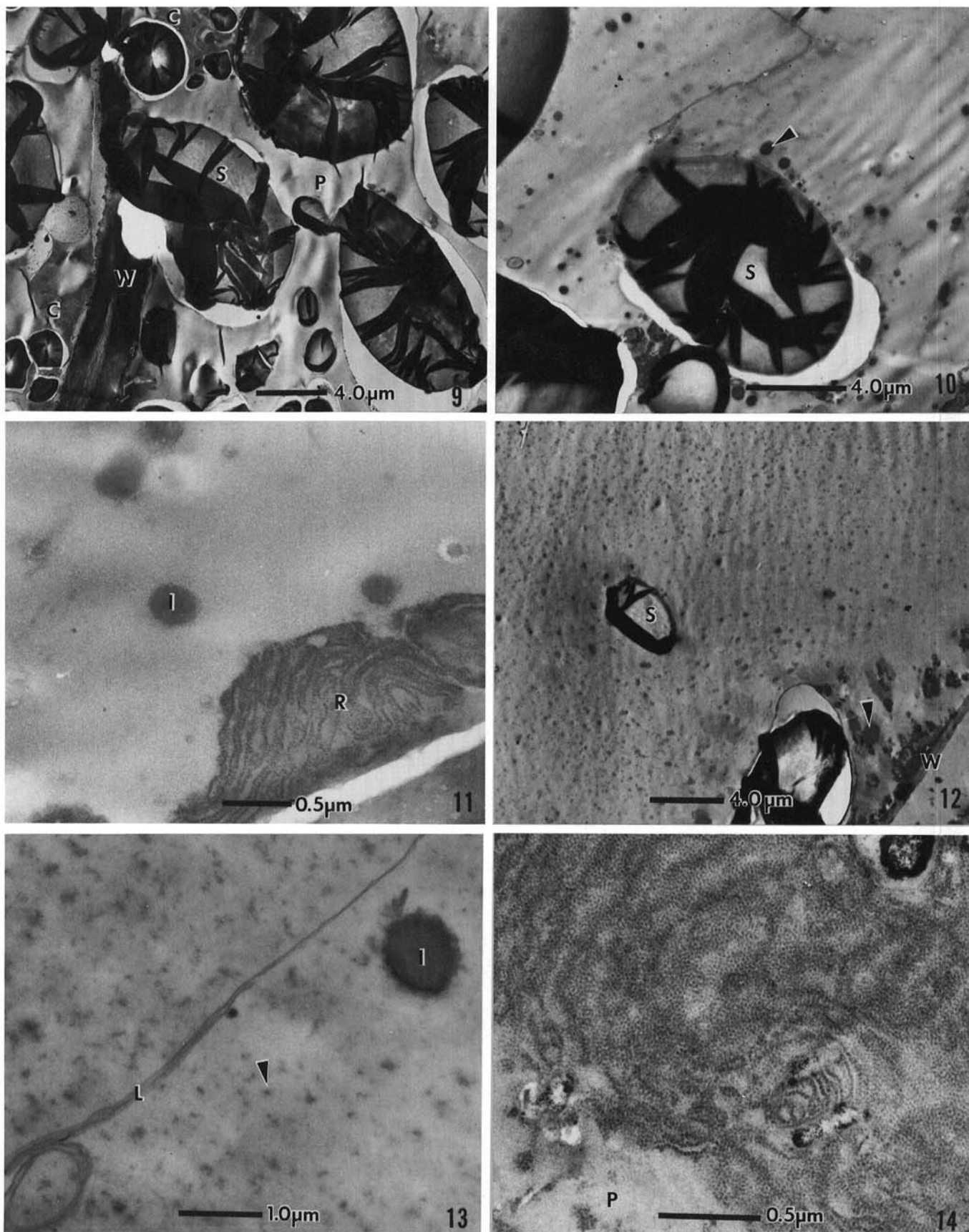


Fig. 9. Low-magnification transmission electron microscopy (TEM) micrograph of Newton showing storage protein (P), starch granules (S), small areas of cytoplasmic remnants (C) and thick cell wall (W) ($\times 3,300$). **Fig. 10.** Low-magnification TEM of Newton showing spherical electron-dense inclusions (arrow) and starch (S) in protein matrix ($\times 4,600$). **Fig. 11.** Cytoplasmic remnants consisted primarily of RER, rough endoplasmic reticulum (R). I = dense inclusion in storage protein ($\times 26,700$). **Fig. 12.** Low-magnification TEM of amphiploid wheat showing numerous small dense inclusions in storage protein. Spherical dense inclusions (arrow) are near thin cell wall (W). Starch granules (S) are small type B granules ($\times 3,300$). **Fig. 13.** High-magnification view of spherical dense inclusion (I), lamellar material (L) and flocculent inclusion (arrow) in storage protein of amphiploid wheat ($\times 15,900$). **Fig. 14.** Cytoplasmic remnants of amphiploid wheat are large and composed of numerous ribosomes and RER-secreted protein (P) ($\times 38,000$).

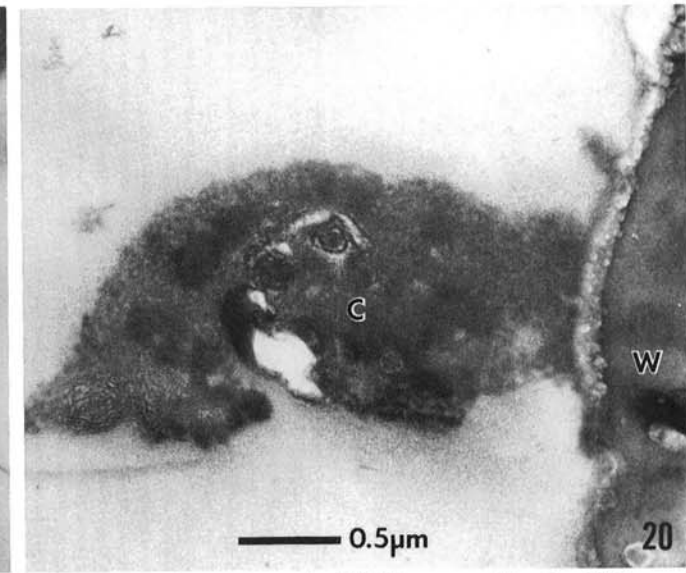
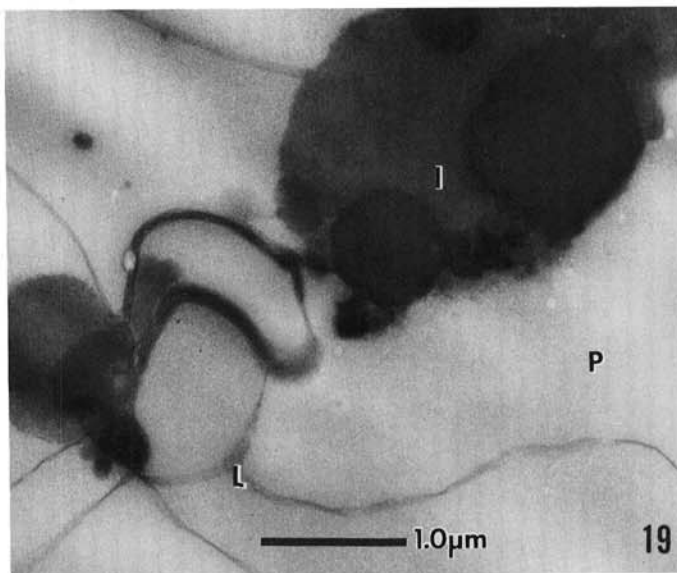
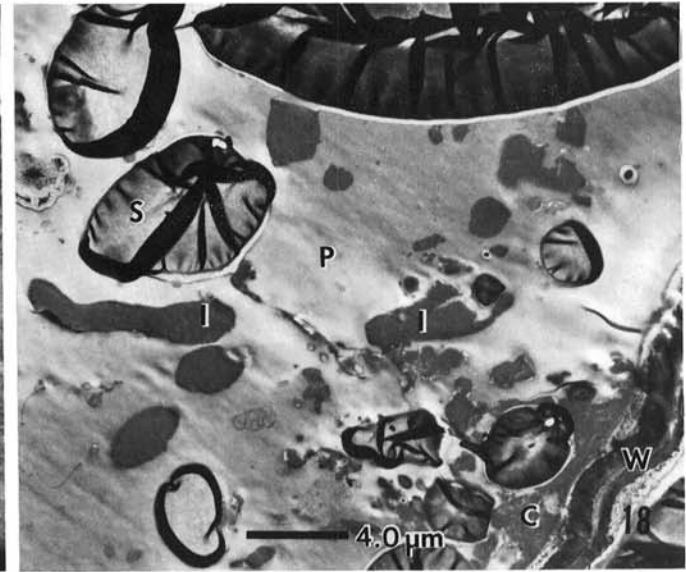
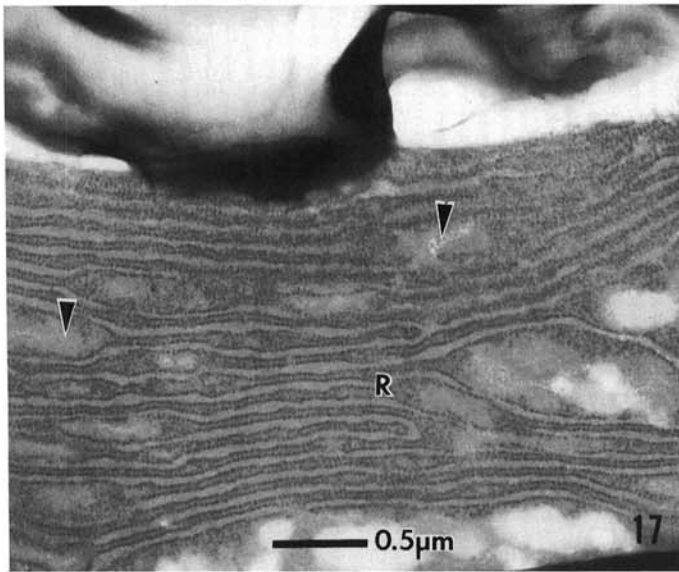
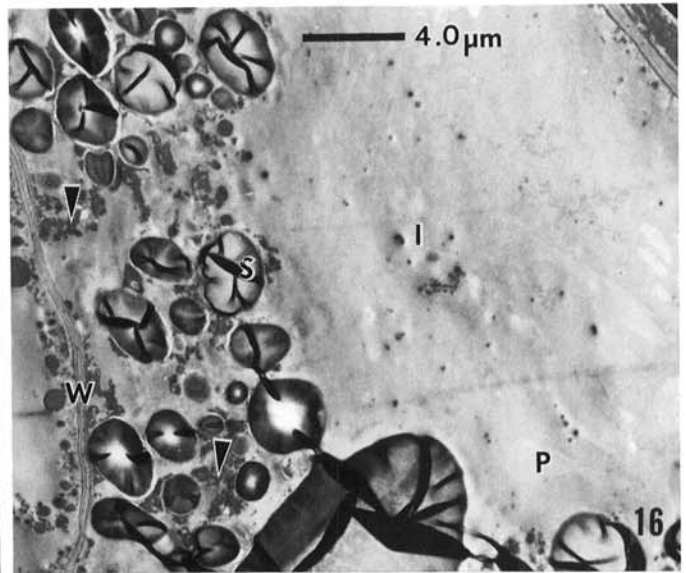
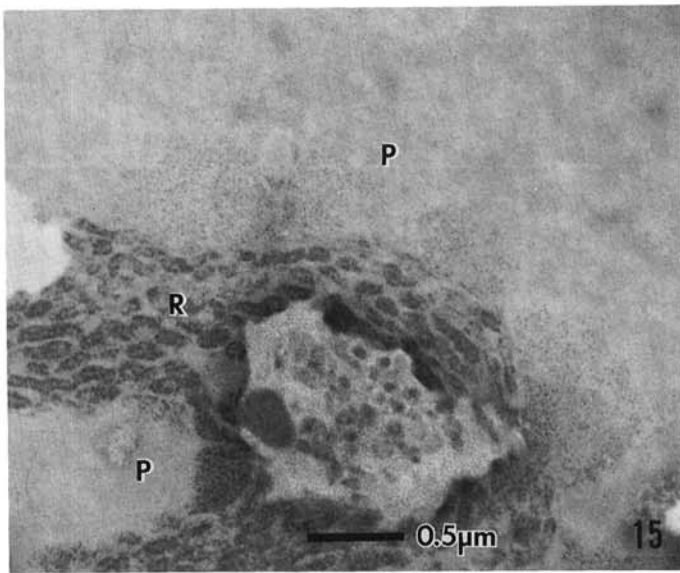


Fig. 15. High magnification of amphiploid cytoplasmic remnants showing large amounts of storage protein (P) being secreted by the RER, rough endoplasmic reticulum (R) ($\times 26,100$). **Fig. 16.** Low-magnification TEM view of tetraploid wheat with small inclusions (I) embedded in storage protein (P). Starch (S) and large irregular inclusions (arrows) are located at cell periphery near the very thin cell wall (W) ($\times 3,300$). **Fig. 17.** Cytoplasmic remnants of tetraploid wheat are large and composed of RER (R). Storage protein is located in the lumen of the RER and within the remnant where the RER membrane has broken (arrows) ($\times 24,500$). **Fig. 18.** Low-magnification TEM view of diploid progenitor showing large inclusions (I) and starch (S) embedded in storage protein (P). Small cytoplasmic remnants (C) are located near the thick cell wall (W) ($\times 3,300$). **Fig. 19.** High-magnification view of storage protein (P) of diploid wheat showing complex inclusion (I) and much lamellar material (L) ($\times 19,600$). **Fig. 20.** Typical cytoplasmic remnant (C) of diploid composed of undifferentiated material near the cell wall (W) ($\times 24,700$).

The tetraploid *T. turgidum* possessed large areas of matrix protein (Fig. 16). The protein of some parts of cells contained very small electron-dense irregular inclusions which were evenly distributed. Most cells showed numerous dense inclusions varying in size and shape that were interspersed among the starch granules (Fig. 16). The cytoplasmic remnants were profuse and were composed of large quantities of RER in which the lumen was filled with material (Fig. 17). The RER membrane apparently ruptured in some places and released some storage protein into the remnant (Fig. 17).

The endosperm of the diploid *T. tauschii*, by contrast, had smaller cells than the other wheats. Large and variously shaped dense inclusions were dispersed in the matrix protein (Fig. 18). The matrix possessed numerous lamellar inclusions, some of which resembled boundaries between adjoining masses of protein (Fig. 19). Cytoplasmic remnants were limited to areas near the cell walls, contained very little discernible RER or ribosomes, and consisted mostly of dense material (Figs. 18–20).

PAGE

PAGE was conducted on 70% ethanol extracts of whole ground kernels to determine relationships among the wheats (Fig. 21). The standard, Marquis (lanes 1, 7), and Newton (lane 2) were very different from the other wheats. *T. tauschii* (lane 3) and *T. turgidum* (lane 5) had unique gel patterns that seemed to have been incorporated into the amphiploid (lane 4). This was confirmed by superimposing the negatives of lanes 3 and 5 to make a double exposure

exposure that yielded lane 6. Note that this double exposure of the two progenitors (*T. tauschii* and *T. turgidum*) gives a gel pattern that is essentially identical to that of the amphiploid (lane 4).

DISCUSSION

It is extremely important to remember when comparing wheats of differing characteristics that a great deal of natural variation exists in each kernel. This normal variation occurs not only from kernel to kernel of the same variety, but also within a single kernel. A region in the starchy endosperm of one high-protein kernel may have more or less protein than the same region of another kernel of the same wheat. Similarly, a low-protein wheat may have a region that exhibits higher protein content than a similar region of a high-protein wheat. The consequence is that large numbers of micrographs must be viewed when comparing wheats of differing characteristics in order to obtain a true representation of these differences (or similarities). Consequently, both a large number of micrographs and an evaluation by image analysis were used in this study to reduce this inherent variation and personal bias as much as possible.

Comparison of the external morphology of the four wheats clearly showed that the amphiploid most closely resembled the tetraploid progenitor, *T. turgidum*. The large size of the amphiploid subaleurone cells and size and distribution of starch also suggested a close affinity to the tetraploid. The appearance of the storage protein of the amphiploid suggested that characteristics of both progenitors had been incorporated into the new wheat. The prominent lamellar inclusions found in the diploid, *T. tauschii* were scattered in the amphiploid storage protein. The small, dense, flocculent inclusions of *T. turgidum* were also present in the amphiploid, however, suggesting another close relationship with the tetraploid. The large amount of cytoplasm (i.e., remnants) in the amphiploid closely resembled that of the tetraploid. The amphiploid RER, however, had released large quantities of protein, a phenomenon that was not found in any of the other wheats. PAGE was used to show that the amphiploid contained the same bands of gliadins as did both *T. tauschii* and *T. turgidum*. PAGE confirmed morphological and light and transmission electron microscopy observations.

Quantitative image analysis clearly has advantages over qualitative personal observations and chemical analyses. Our results show that despite the large variation within and among the wheats, image analysis closely parallels chemical analyses for storage proteins (Tables I, II). While the staining procedures used in this study cannot quantify the amount of protein, the numbers generated by the image analyses lead to the same results as chemical analyses (i.e., *T. turgidum* has the highest protein content followed by the amphiploid, *T. tauschii*, and Newton). Image analysis of micrographs can depict the amount of variation, as well as quantitatively show the distribution of starch and protein within different regions and different wheats. This method should prove useful when comparing new wheats to determine where the storage protein is located and how it is distributed.

The results of this structural study show that the new hexaploid wheat closely resembles the primitive wheats from which it was created. This synthetic hexaploid (amphiploid) wheat had few characteristics representative of currently cultivated hexaploid cultivars (e.g., Newton). We have shown that microscopic and biochemical techniques can be used to elucidate structural and compositional differences among various wheats. Microscopy should prove useful in elucidating structural changes as other procedures are developed for the transfer of genetic information into bread wheats.

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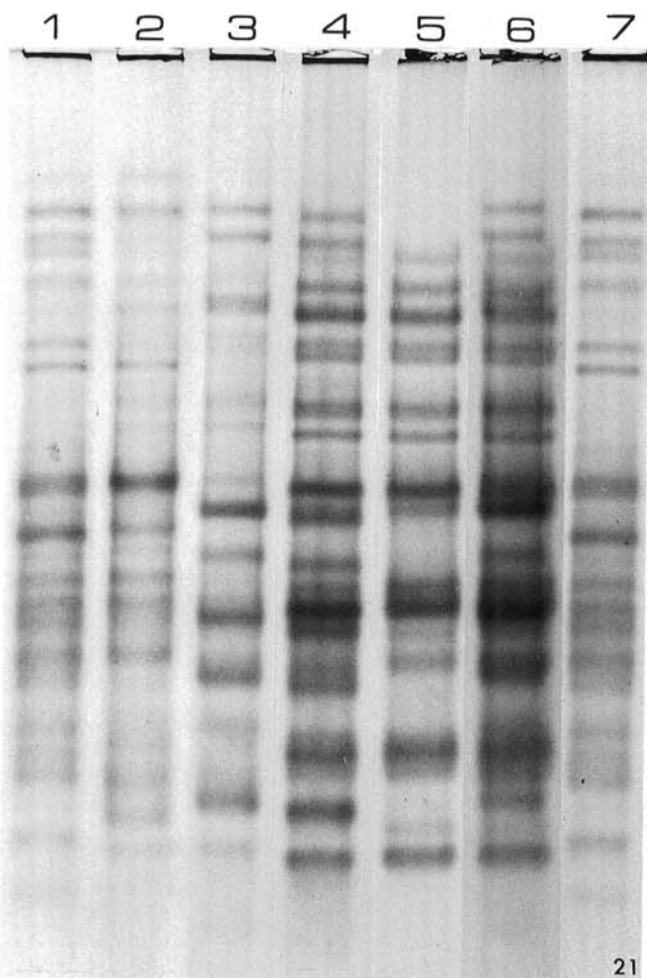


Fig. 21. Polyacrylamide gel of 70% ethanol extract of ground kernels. Lanes 1 and 7, Marquis standards; lane 2, Newton; lane 3, *Triticum tauschii*; lane 4, amphiploid; lane 5, *T. turgidum*. Lane 6 was produced photographically by superimposing negatives of lanes 3 and 5 to yield a double exposure. Note that the appearance of lane 6 is nearly identical to that of the amphiploid, lane 4.

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