A Freeze-Fracture Study of Storage Protein Accumulation in Unfixed Wheat Starchy Endosperm

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ABSTRACT

A freeze-fracture study of storage protein accumulation was conducted on developing endosperm of hard red winter wheat. Storage protein was observed in the starchy endosperm as discrete membrane-bounded protein bodies about seven days after flowering (DAF). The protein bodies fused with vacuoles and deposited the storage protein into the vacuoles. Also present in the cytoplasm at 7 DAF were numerous Golgi bodies that were apparently connected to large sheets of rough endoplasmic reticulum (RER). Protein bodies were greatly enlarged by 12 DAF and very complex with numerous vesicles associated with their periphery. The RER had changed from large sheets to small cisternal elements that were interconnected by tubular ER. Small vesicles were produced by the ER. By 17 DAF storage protein matrix was evident. The matrix, consisting of large cross-fractured areas of protein, was membrane bounded and in close association to both starch granules and cytoplasm. The matrix enlarged during maturation (19–35 DAF), as did the starch granules, and resulted in isolating the cytoplasm into small regions of the endosperm cell.

In numerous studies centering on the formation of the starchy endosperm storage proteins of wheat a variety of mechanisms of protein body formation have been formulated. Some of these mechanisms, such as the proteoplast theory (Morton and Raison 1963, Morton et al 1964) and a theory concerning free ribosomes within vacuoles (Barlow et al 1974), are not substantiated. The suggestion of Buttrose (1963) that the Golgi apparatus is involved in storage protein deposition has at least circumstantially been confirmed (Parker 1981, 1982; Parker and Hawes 1982; Bechtel and Gaines 1982; Bechtel et al 1982a). Recently, it was hypothesized that prolamin-containing protein bodies in wheat are not vacuolar in origin, but that the protein bodies form in ER that subsequently ruptures and results in protein granules not completely enclosed by a membrane (Miflin et al 1983). This hypothesis is in contrast to convincing microscopical evidence for the vacuolar location of protein bodies as well as the involvement of the Golgi apparatus in protein body formation (Parker 1980, 1981, 1982; Parker and Hawes 1982; Bechtel and Gaines 1982; Bechtel et al 1982a,b; Bechtel and Barnett 1986). This paper uses the terminology of Bechtel et al (1982a), which defines a protein body as a discrete granule of storage protein surrounded by a single trilaminar membrane; therefore, a protein body can have single or multiple granules, and a vacuole containing protein granules can be considered a protein body.

Much of the controversy in interpreting the origin of protein bodies arises from difficulty in deciding what structural features represent artifacts in chemically fixed material (Bechtel and Barnett 1986; Bechtel 1983; Miflin et al 1983). We developed and evaluated a freeze-fracture technique whereby replicas of unfixed wheat endosperm were obtained that had near lifelike preservation (Barnett and Bechtel 1984, Bechtel 1985, Bechtel and Barnett 1984a,b). This paper reports the results of a freeze-fracture study on development of wheat starchy endosperm protein bodies and subsequent formation of the protein matrix. These results substantiate our previous observations that protein bodies are membrane bounded from their inception to deposition into vacuoles, at which time the tonoplast forms the bounding membrane.

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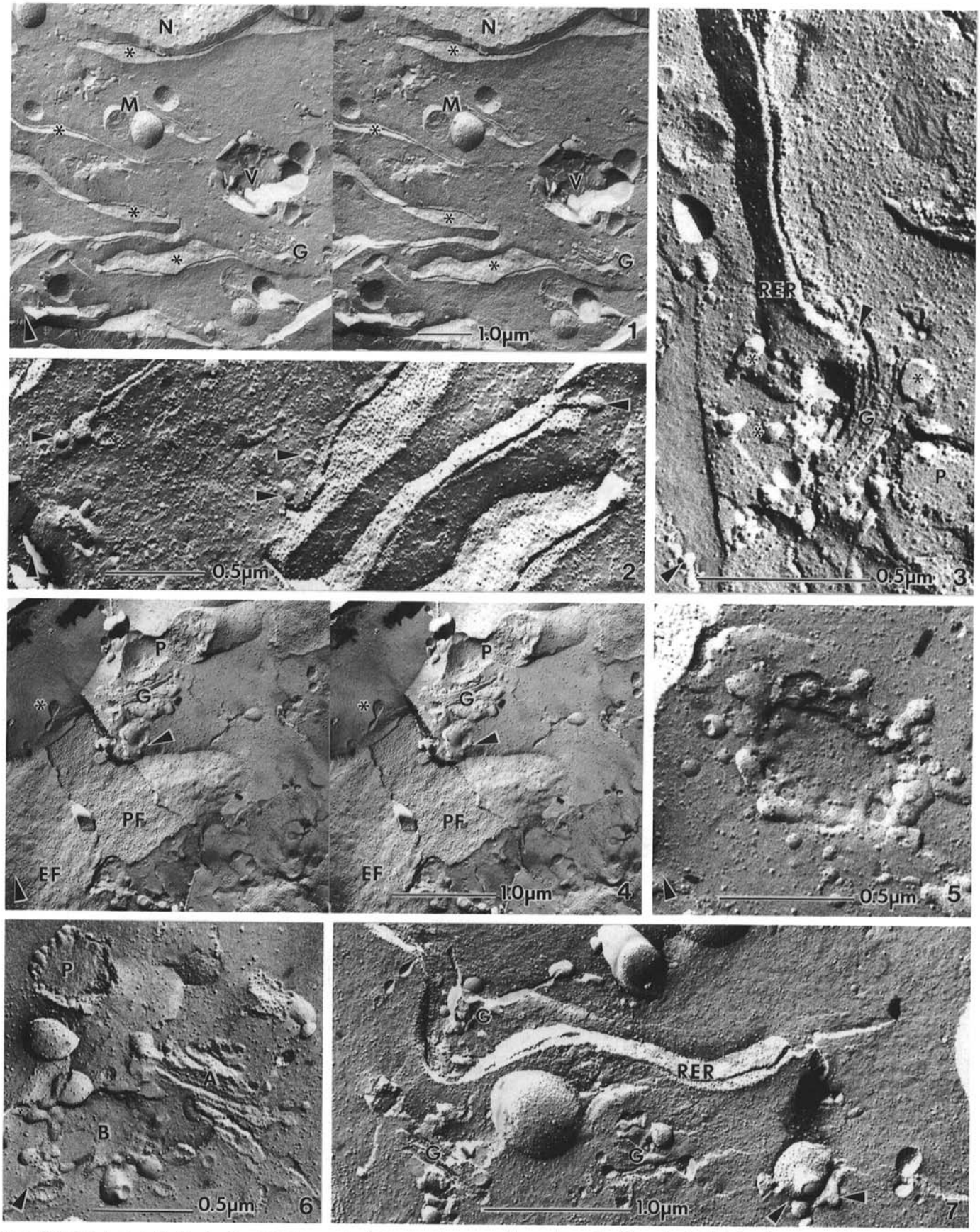
MATERIALS AND METHODS

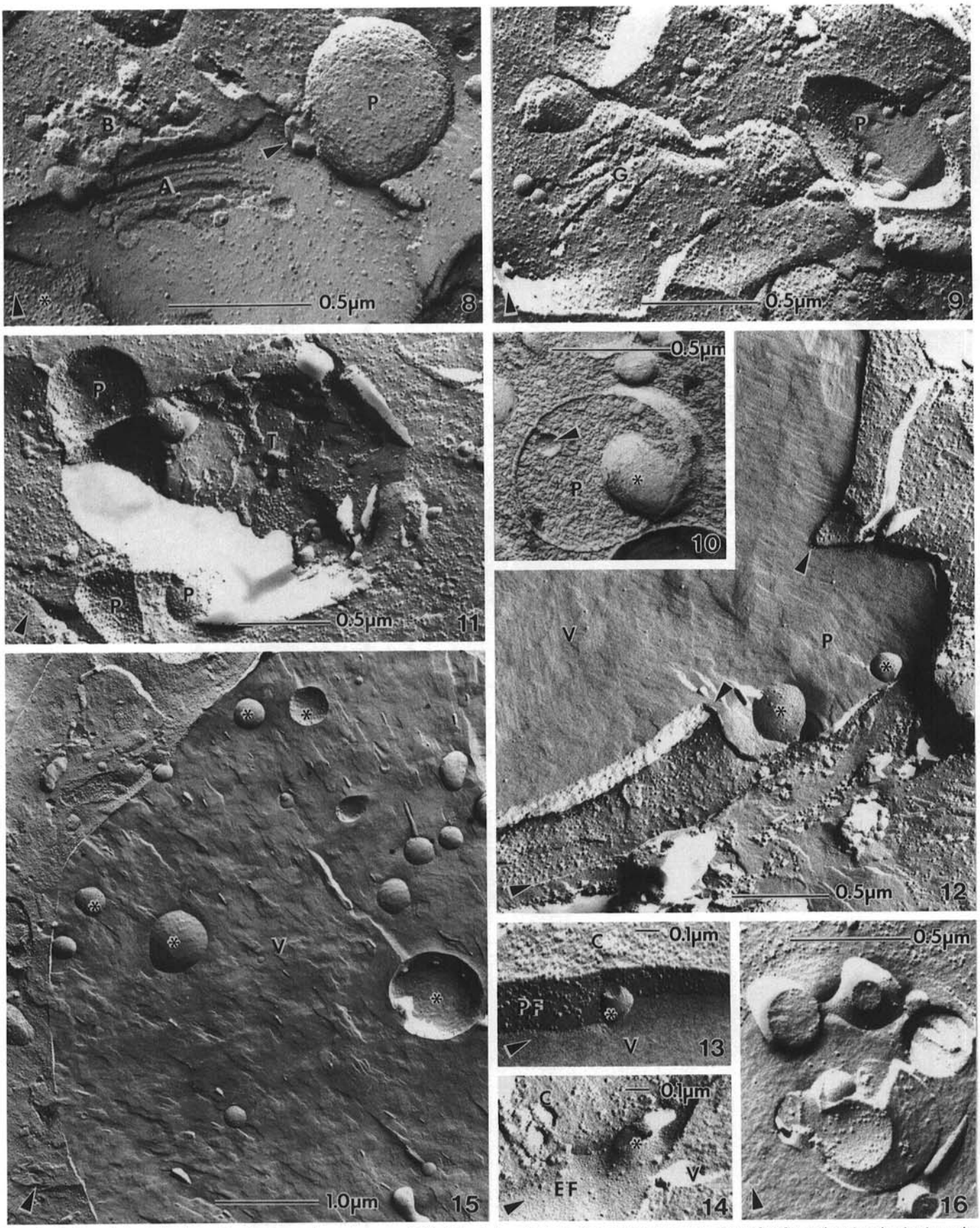
Hard red winter wheat *Triticum aestivum* cv. Newton was grown in experimental plots near Manhattan, KS, during the 1982 and 1983 growing seasons. Caryopses were tagged at the time of flowering and harvested at seven, 12, 14, 17, 21, and 28 days after flowering (DAF). Samples to be freshly frozen were cut into 1–2-mm thick cross-sections and thrown into liquid nitrogen (LN_2)-cooled monochlorodifluoromethane (Freon-22). The frozen endosperm pieces were tranferred to cryogenic vials and stored under LN_2 until needed. Unfixed glycerol-imbibed tissue was prepared by harvesting entire wheat panicles two days before the sampling date, with 15 cm of stem attached, and immediately immersing the cut stem into 20% glycerol. The heads were maintained on 20% glycerol for 48 hr on a sunny windowsill in the laboratory. On the sample date, individually tagged caryopses were removed, cut into several large pieces, frozen, and stored as described for the freshly frozen tissue.

Freeze-fracture was conducted on 12-, 14-, 21-, and 28-DAF freshly frozen tissue by fracturing under LN_2 until pieces of tissue obtained were small enough to fit into the specimen holders. The holders were placed on a cold stage at −20°C; the frozen endosperm was placed in the holder, and secured with cold 30% glycerol (4°C), which froze quickly. Visible specimen thawing did not occur under these conditions, although some recrystallization probably occurred. The temperature of the specimen was immediately lowered to that of the LN_2-cooled fluorocarbon. Glycerol-imbibed unfixed tissue was prepared for freeze-fracture by fracturing into small pieces under LN_2. These fragments were then thawed in 30% glycerol, trimmed with a new razor blade to reveal the aleurone and subaleurone regions, placed in the holders, and refrozen in LN_2-cooled fluorocarbon. This procedure was shown by Rash (1979) not to induce excessive damage.

Replicas of the variously prepared samples were made in a modified Denton DFE-3 freeze-etch apparatus on a Denton DV 502 vacuum evaporator using the apposed specimen tooling and gold holders. Specimens were deiced at −110°C and fractured at −150°C at 1×10^{-6} torr or better vacuum with a cold shroud at LN_2 temperature surrounding the specimens. Resistance evaporation of platinum and carbon immediately followed specimen fracturing. Some samples were etched at −100°C before replication but were found to lose much ground plasmic detail; therefore, etching was not routinely used. Replicas were cleaned in sulfuric acid, dichromate cleaning solution, sodium hypochlorite, or various sequential combinations of these agents. No single cleaning sequence worked consistently. Replica breakup of late-stage endosperm was reduced by placing small pieces of filter paper beside the specimen before freezing. Apparently the paper holds the endosperm together as the starch granules swell during acid

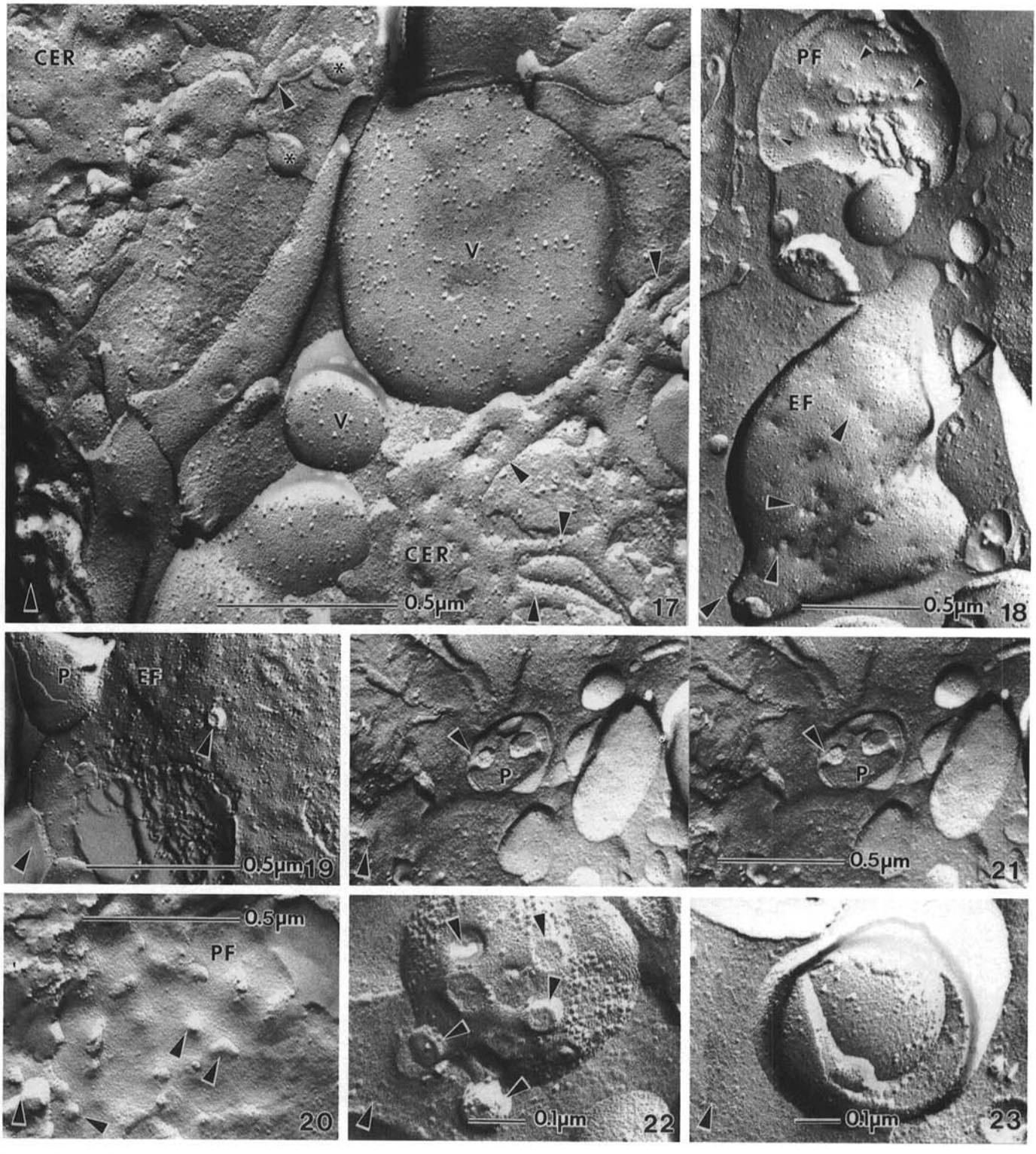


Figs. 1–7. Protein body initiation in wheat. **1,** Stereo pair of wheat endosperm cell seven days after flowering (DAF) showing sheets of rough endoplasmic reticulum (RER) (*), vacuole (**V**), Golgi body (**G**), nucleus (**N**), and mitochondria (**M**) (×9,900). **2,** Enlargement of Fig. 1 depicting vesicles (**arrows**) forming from RER (× 37,100). **3,** Golgi body (**G**) connected (**arrow**) to RER. Note numerous Golgi vesicles (*) and cross-fractured protein body (**P**) (× 64,000). **4,** Stereo pair of 7-DAF endosperm showing the endoplasmic leaflet (**EF**) and protoplasmic leaflet (**PF**) of the RER. Golgi body (**G**) appears to be connected to RER by fenestrated structure (**arrow**). Complex protein body with inclusions (**P**) lies near Golgi body. Note vesicle (*) forming from endoplasmic face (**EF**) of RER (× 19,100). **5,** Golgi body in 7-DAF endosperm budding numerous vesicles (× 51,300). **6,** Two Golgi bodies in 7-DAF endosperm, one in vertical fracture (**A**), the other in horizontal fracture (**B**) budding numerous vesicles. Note cross-fractured protein body (**P**) (× 36,900). **7,** Three Golgi bodies (**G**) associated with RER in 7-DAF endosperm. Note that several small vesicles are fused with protein body (**arrows**) (× 28,000).

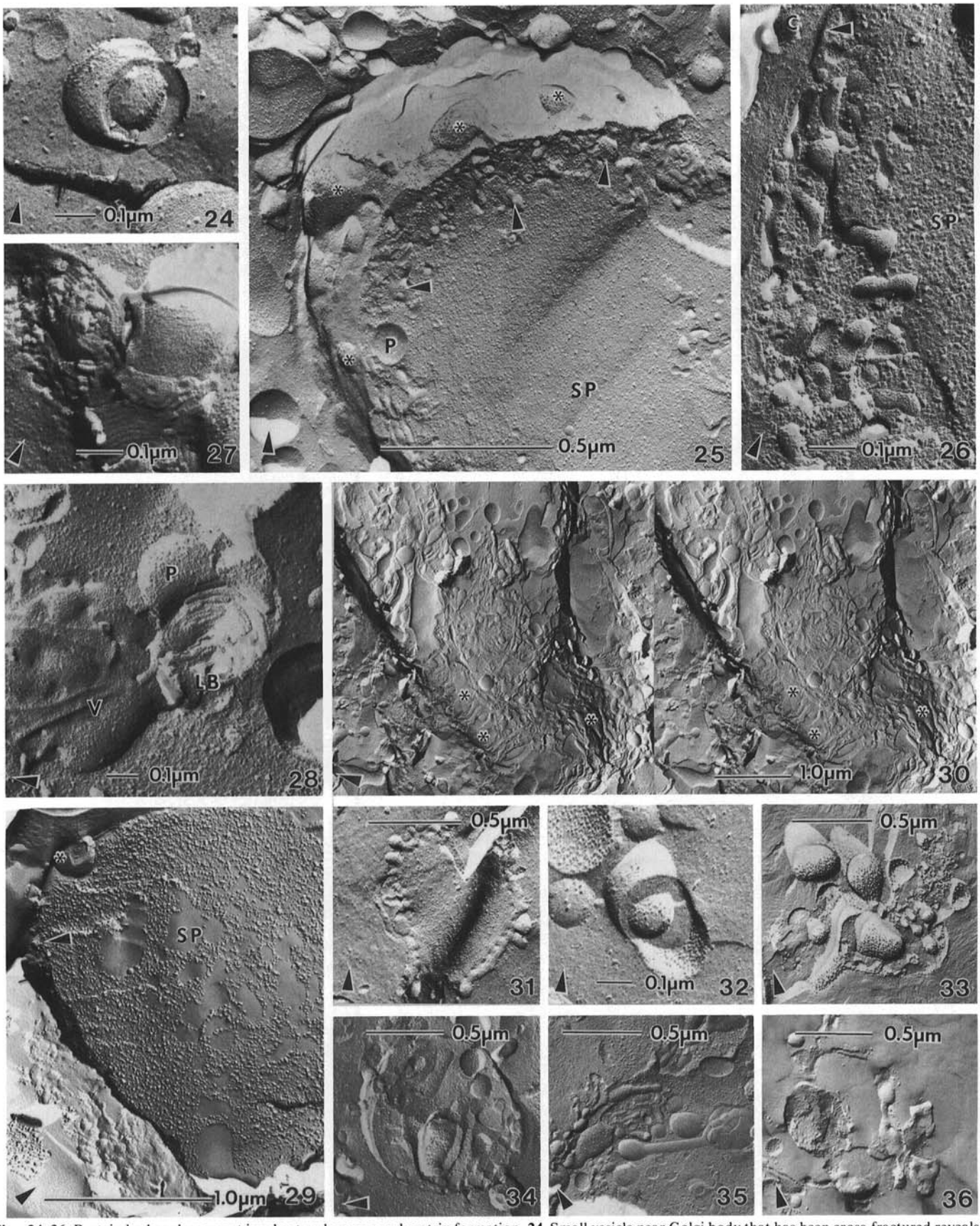


Figs. 8–16. Protein body initiation in wheat starchy endosperm. **8,** Golgi body in wheat starchy endosperm seven days after flowering showing both vertical (**A**) and horizontal (**B**) fracture planes associated with protoplasmic face of rough endoplasmic reticulum RER (*). Protein body (**P**) has several vesicles associated or fused with tonoplast (**arrow**) (× 55,000). **9,** Cross-fracture through protein body (**P**) showing deposits. Golgi body (**G**) and vesicles are in close association with protein body (×43,700). **10,** Cross-fractured protein body (**P**) that was etched before replication reveals large protein granule (*) and small granule (**arrow**) (× 44,000). **11,** Enlargement of vacuole in Fig. 1 showing several protein bodies (**P**) fused to tonoplast and irregular nature of tonoplast (**T**) (×33,600). **12,** Cross-fracture through vacuole (**V**) and protein body (**P**) exhibit fused membranes (**arrows**) and protein granules (*) within protein body (×36,800). **13,** Protoplasmic face (**PF**) of vacuole (**V**) tonoplast with fused vesicle (*); **C** = cytoplasm (× 44,100). **14,** Endoplasmic face (**EF**) of vacuole tonoplast (**V**) with fused vesicle (*); **C** = cytoplasm (× 44,100). **15,** Cross-fracture through portion of large central vacuole (**V**) showing numerous protein granules (*) (×19,000). **16,** Protein granules within a portion of a vacuole showing granule fusion (× 45,500).

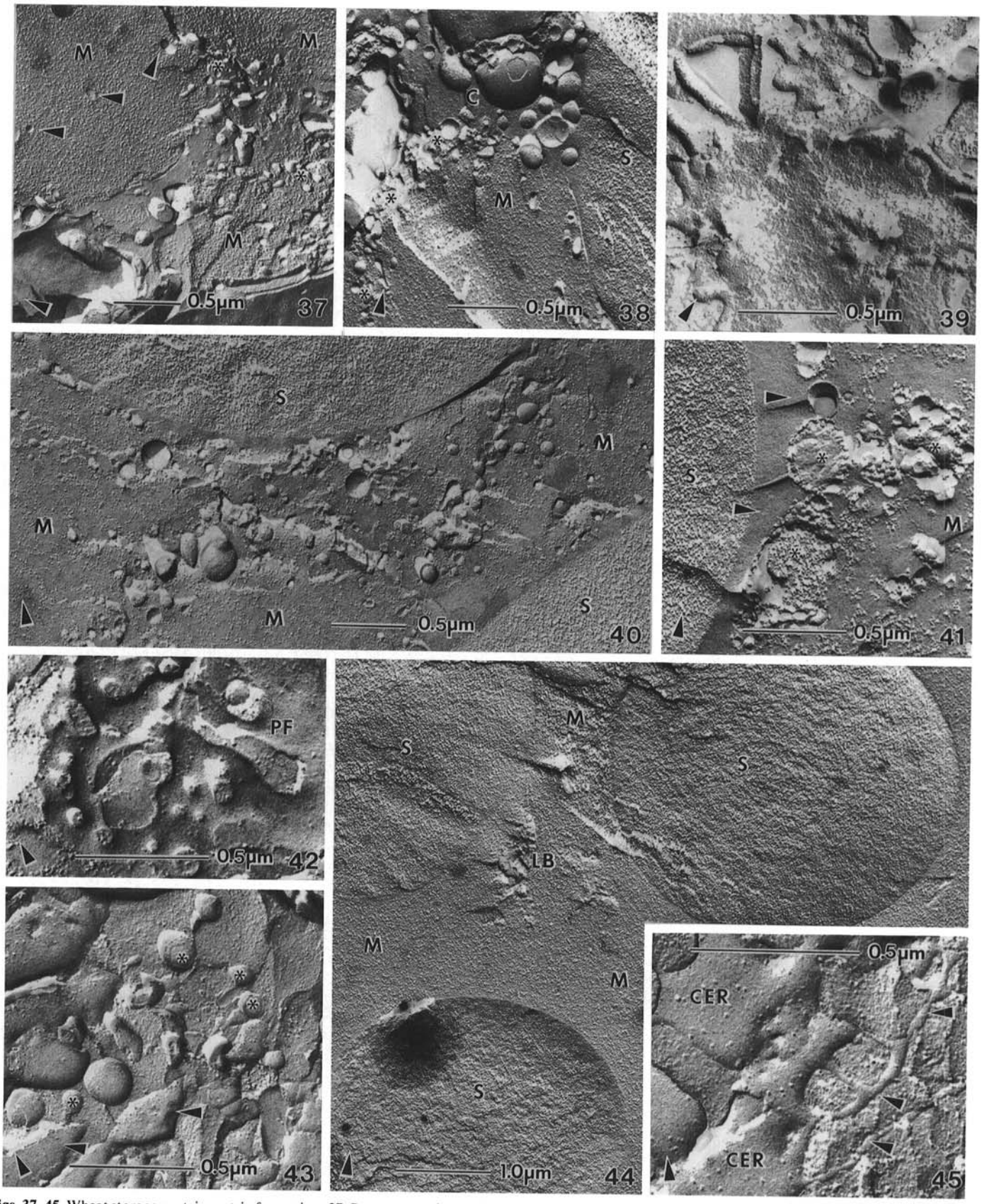
hydrolysis and reduces replica breakup. Replicas were viewed in a Philips EM 201 transmission electron microscope (TEM) at 60 kV. Stereo pairs were taken at ±6° from incident illumination. Electron micrographs of replicas are reproduced without reversal, with platinum electron dense (with white shadows). The direction of shadowing is indicted on each freeze-fracture micrograph by an arrow in the lower left corner. Freeze-fracture terminology is after Branton et al (1975).



Figs. 17–23. Protein body enlargement in wheat starchy endosperm. **17,** Portion of a large region of endoplasmic reticulum (ER) in wheat starchy endosperm 12 days after flowering showing cisternal elements (**CER**) interconnected to tubular ER (**arrows**). Note vesicles (*****) forming from ER. Vesicles (**V**) may be protein bodies (× 64,300). **18,** Vacuole-like structures exhibiting depressions (**arrows**) on the endoplasmic face (**EF**) and bulges (**small arrows**) on the protoplasmic surface (**PF**) (× 41,400). **19,** Endoplasmic face (**EF**) of vacuole from freshly frozen wheat endosperm showing volcano-like depression (**arrow**). Note association of probable protein body (**P**) with tonoplast (**EF**) (× 55,800). **20,** Protoplasmic face (**PR**) of tonoplast reveals bulges in membrane (**arrows**) (× 55,800). **21,** Stereo pair of a cross-fractured protein body (**P**) exhibiting pinocytosis (endocytosis, **arrow**) (× 45,400). **22,** Oblique fracture around protein body endoplasmic face showing the numerous small vesicle fusions (**arrows**) (× 103,000). **23,** Cross-fracture through protein body showing large and small protein granules (× 80,900).



Figs. 24–36. Protein body enlargement in wheat endosperm and matrix formation. **24,** Small vesicle near Golgi body that has been cross-fractured reveals granule within (× 76,400). **25,** Fracture through vacuole shows irregular nature of endoplasmic face of tonoplast where vesicle fusions have taken place (*) that deposited a protein granule (**P**). Storage protein (**SP**) within vacuole has irregular and vesicular periphery (**arrows**) (× 31,700). **26,** Enlargement of cross-fracture through vacuole containing storage protein (**SP**) showing vesicular periphery inside the irregular tonoplast (**arrow**); **C** = cytoplasm (× 83,900). **27,** Lamellar body associated with periphery of vacuole (× 83,800). **28,** Lamellar body (**LB**) associated with protein body (**P**) and portion of vacuole (**V**) (×63,700). **29,** Cross-fractured vacuole from freshly frozen wheat endosperm with ice-damaged granular storage protein (**SP**), irregular periphery (**arrow**), and small lamellar body (*) (× 30,600). **30,** Stereo pair of wheat endosperm 17 days after flowering (DAF) with large region of endoplasmic reticulum (**ER**) showing cisternal ER (*) interconnected by tubular ER. Note the large number of vesicles produced by the ER (× 14,700). **31,** Golgi body in 17-DAF wheat endosperm (× 38,200). **32,** Cross-fracture through small vesicle of 17-DAF wheat endosperm showing granule of protein (× 61,500). **33,** Complex protein body from freshly frozen wheat endosperm (× 29,600). **34,** Complex protein body in endosperm of 17-DAF wheat (× 29,600). **35,** Vesicles and tube-like structures prevalent in 17-DAF wheat endosperm (× 29,600). **36,** Tube-like structure in freshly frozen wheat endosperm (× 29,600).



Figs. 37–45. Wheat storage protein matrix formation. **37,** Storage protein matrix (**M**) of endosperm 17 days after flowering (DAF) had occasional inclusions (**arrows**). Note vesicular material at periphery (*) (× 26,400). **38,** Portion of starch granule (**S**) is surrounded by matrix protein (**M**) and cytoplasm (**C**). Storage protein (**M**) has numerous vesicles at its periphery and between the cytoplasm (**C**) (× 26,000). **39,** Cisternal and tubular ER in 21-DAF freshly frozen wheat endosperm (× 36,400). **40,** Starch (**S**) is partially surrounded by matrix storage protein (**M**) in 21-DAF endosperm, but vesicular material is prominent (× 28,000). **41,** Twenty-eight-DAF wheat endosperm lacks much of the vesicular material associated with the matrix in earlier stages of development. The matrix protein (**M**) has several types of inclusions (*), and a space (**arrows**) still exists around starch granules (**S**) (× 39,800). **42,** Protoplasmic face (**PF**) of 28-DAF wheat endosperm tonoplast still exhibits irregular surface indicative of vesicle fusions and endocytosis (× 50,100). **43,** Endoplasmic reticulum (**arrows**) of 28-DAF wheat endosperm has numerous vesicles (*) associated with it (× 46,500). **44,** Endosperm of mature wheat has starch granules (**S**) completely surrounded by matrix protein (**M**) without spaces around the granules (**S**). Compressed lamellar bodies can be observed (**LB**) (× 18,000). **45,** Cisternal (**CER**) and tubular endoplasmic reticulum (**arrows**) are present in mature endosperm (× 62,900).

RESULTS

Protein Body Initiation

Freeze-fracture of glycerol-imbibed, 7-DAF wheat starchy endosperm reveals cells with a cytoplasm containing large sheets of rough endoplasmic reticulum (RER), Golgi bodies, a nucleus, and a variety of membrane-bounded structures (Fig. 1). Small vesicles are in association with the large sheets of RER (Fig. 2). The small size of these vesicles made it difficult to determine if they were derived from or going to the ER, but their tear-drop shape suggests that they are formed from the RER (Fig. 2). Closely associated with the RER and possibly directly connected to it are Golgi bodies (Figs. 3 and 4). The Golgi apparatus from 7-DAF endosperm typically possessed numerous vesicles at the periphery of the cisternae (Figs. 5 and 6). Small vesicles, possibly from Golgi bodies or RER, apparently fuse with other larger vesicles (protein bodies; Figs. 7 and 8) and deposit the contents inside (Figs. 9 and 10). Etching of some specimens at −100°C for 15 sec revealed these deposits within the protein body near the surface of the protein granules (Fig. 10). Spherical protein bodies are fused to vacuoles (Fig. 11) and protein granules are deposited into the vacuole (Fig. 12). The tonoplasts of vacuoles exhibit irregularities in the membrane indicative of small vesicle fusions (Figs. 13 and 14). Cross-fractures through the large central vacuoles of 7-DAF wheat endosperm cells reveal scattered protein granules (Fig. 15), some of which (i.e., the smaller protein granules) are in various stages of fusion (Fig. 16).

Protein Body Enlargement

Twelve-DAF cytoplasm appears much different than cytoplasm at 7 DAF. The RER at 12 DAF has been reorganized from the large sheets (Fig. 1) to small cisternal elements with interconnected tubular ER producing small vesicles (Fig. 17). The ER is associated with large spherical vesicles (protein bodies), but direct connections between the two were never observed (Fig. 17).

Another feature of the 12-DAF cytoplasm is the presence of a membrane system (vacuoles) characterized by endocytic vesicles on both endoplasmic (EF) and protoplasmic (PF) membrane faces (Fig. 18). The convex EF membrane leaflet possessed numerous depressions (pits) whereas the concave PF surface exhibited bulges (Fig. 18). Similar membrane systems were also observed in freshly frozen wheat endosperm samples (Figs. 19 and 20). Rarely, the membranes would cross-fracture in a manner that revealed both the endocytotic vesicle and its attachment to the tonoplast (Fig. 21). Oblique fractures of small spherical protein bodies show numerous vesicle fusions and associations (Fig. 22), whereas cross-fractures through these structures reveal various-sized protein granules within the vesicle membrane (Figs. 23 and 24).

These protein bodies are found closely associated with vacuoles where the irregular tonoplast indicate fusion between protein bodies and vacuoles (Fig. 25). Inside the vacuoles are masses of cross-fractured protein having a homogeneous center and a very irregular periphery (Figs. 25 and 26). Associated with the periphery of many vacuoles at 12 DAF are lamellar bodies (Figs. 27 and 28). Freshly frozen wheat also exhibits vacuolar protein deposits that have granular cross-fractured texture, peripheral lamellar bodies, and vesicles (Fig. 29).

Protein Matrix Formation

By 17 DAF storage protein matrix is evident. The cytoplasm at this stage of development contains ER similar to that of 12-DAF wheat endosperm. Small cisternal elements are continuous with tubular ER (Fig. 30), but there are a larger number of vesicles being produced by the 17-DAF tubular ER than that of 12-DAF ER (Figs. 17 and 30). Golgi bodies in 17-DAF endosperm are not as complex as at earlier stages, but each cisternum is budding large numbers of small vesicles (Fig. 31). The cytoplasm contains many small simple protein bodies (Fig. 32) in addition to larger more complex ones that show signs of vesicle fusion as well as endocytosis in both freshly frozen and glycerol-imbibed tissues (Figs. 33 and 34). Also prevalent in the cytoplasm at this stage of starchy endosperm development are small regions of tubes and vesicles independent of the ER (Fig. 35). These tubes seem to produce numerous vesicles and are found in both glycerol-treated and freshly frozen endosperm tissues (Figs. 35 and 36).

The matrix protein of 17 DAF wheat endosperm consists of large cross-fractured areas of protein (Fig. 37) in close association to both starch granules and cytoplasm (Fig. 38). Much of the matrix is homogeneous with occasional inclusions (Fig. 37). The periphery of the matrix next to the tonoplast is irregular as is the periphery of large protein bodies of 12-DAF endosperm (Figs. 26, 37, and 38). Although cross-fractures of both protein matrix and starch granules have a similar appearance, the starch granules always have a smooth edge while that of storage protein is irregular (Fig. 38).

Twenty-one-DAF wheat endosperm has cytoplasm similar to that of 17-DAF wheat. Both glycerol-imbibed (micrographs not shown) and freshly frozen 21-DAF cytoplasm exhibit cisternal and tubular ER (Fig. 39). Matrix protein in 21-DAF endosperm is located between starch granules, with vesicular material associated with it (Fig. 40). These vesicles disappear by 28 DAF (Fig. 41). Small spaces between starch granules and matrix protein are also observed at 28 DAF (Fig. 41). The tonoplast surrounding the matrix, however, continues to exhibit an irregular surface suggestive of vesicle fusions (Fig. 42). Cisternal and tubular ER with associated vesicles are also apparent in 28-DAF cytoplasm (Fig. 43). At maturity, the matrix protein is pressed closely to the starch granules (Fig. 44). Much of the vesicular material associated with the matrix at earlier stages is not observed and only a few inclusions that look like compressed lamellar bodies are present (Fig. 44). Small regions of mature cytoplasm are devoid of vesicles but contain both tubular and cisternal ER (Fig. 45).

DISCUSSION

The freeze-fracture of glycerol-imbibed wheat endosperm used in this study previously was evaluated and found to yield replicas of excellent quality that correlate well with freshly frozen samples and induce few artifacts affecting interpretation of protein body formation (Bechtel and Barnett 1986). With this technique we were able to follow wheat endosperm development without the use of chemical fixatives. The results we obtained with freeze-fracture of both glycerol-imbibed and freshly frozen wheat endosperm parallel some of the results obtained from conventionally prepared samples for TEM (Parker 1981, 1982a,b; Bechtel et al 1982a,b). The results of this report and our freeze-fracture evaluation (Bechtel and Barnett 1986), however, do not support the contention that protein bodies in wheat starchy endosperm are not membrane bounded throughout development (Miflin et al 1983) or that protein bodies are connected directly to the RER (Campbell et al 1981).

Our results indicate that there are two major factors that can affect interpretation of electron micrographs. The first is the dramatic change that starchy endosperm undergoes during morphogenesis. Early in development the endosperm was a nondescript undifferentiated thin layer of coenocytic cytoplasm that differentiated into highly vacuolated cells with cell walls within two days (Bechtel et al 1982a). These cells then underwent further complex changes such as the transformation of the large RER sheets into small cisternal elements interconnected by tubular ER. In addition, the tonoplast of vacuoles became irregular, making interpretations from standard thin sections difficult. This irregularity may be responsible for some concluding that there is no bounding membrane (Miflin et al 1983). Late in development these vacuoles fused with one another in a phenomenon that produced the protein matrix that eventually surrounded starch granules and cytoplasmic remnants (Bechtel et al 1982b). Freeze-fracture of freshly frozen samples clearly showed that the tonoplast is intact at least until physiological maturity. Couple these complex changes with fixation effects, the second major contributing factor to micrograph interpretation, and it is easy to understand why so many different mechanisms of protein body formation have been proposed. Fixation effects have never been thoroughly investigated. Jennings, Morton, and Palk (1963) concluded that osmium tetroxide fixation causes protein granules to shrink away from the tonoplast. They also attributed membrane disruption, caused by osmium tetroxide, to multiple protein granules within vacuoles as well as the production of membranous inclusions. A

variety of fixation schedules have been used to investigate wheat protein body inclusions (Parker 1980), but none seems to affect the inclusions. We have processed numerous wheat samples for electron microscopy and found that preservation quality is highly dependent upon mechanical damage (Bechtel 1983). We have come to appreciate just how many different views of wheat endosperm can be produced using traditionally prepared tissues. Thus, our approach to understanding the structural aspects of wheat protein body formation was to develop the freeze-fracture technique to look at unfixed samples. This technique eliminated many of the problems associated with preparing developing wheat endosperms for microscopy.

There are several salient results from this freeze-fracture study. First is the conversion of the large sheets of RER typical of 7-DAF endosperm into small cisternal elements (CER) interconnected by tubular ER (TER) found during later stages of protein body secretion. Whereas the interconnections between cisternal and tubular ER have previously been shown with high-voltage electron microscopy (Parker and Hawes 1982), neither the conversion of RER sheets into CER and TER nor the production of vesicles from ER have been depicted for developing wheat endosperm before. The association of Golgi bodies to ER and the suggestion that dictyosomes participate in protein packaging in developing wheat are not new observations and ideas. Buttrose (1963) originally suggested Golgi body involvement in protein body secretion; however, this has been dismissed by some (Barlow et al 1974, Briarty et al 1979, Miflin et al 1983). A series of studies has shown the presence of Golgi bodies throughout wheat endosperm development (Campbell et al 1981; Parker 1981, 1982; Bechtel et al 1982a,b) as well as the sensitivity of Golgi vesicles to proteases (Bechtel and Gaines 1982) and the tremendous numbers of Golgi vesicles in endosperm cells (Parker and Hawes 1982).

The question still unanswered is how the storage proteins get to the Golgi apparatus from the ER, their site of synthesis. In some protein-secreting plant cells there are direct connections between RER and dictyosomes (Juniper et al 1982). Parker and Hawes (1982) could not show conclusively direct connections nor were they able to demonstrate ER-derived vesicles. We reconstructed serial sections and found direct connections to the Golgi cisternae and CER by TER (*unpublished*). Our freeze-fracture micrographs also depict similar connections. It is doubtful that the RER-formed vesicles participate in the transport of proteins to the Golgi apparatus. They are of similar size as Golgi-derived vesicles, but such vesicles were never observed near the forming faces of Golgi bodies. We, therefore, think that the direct connections between RER and dictyosomes provide the major avenue of transport. The RER-derived vesicles probably function in protein body enlargement and provide an alternative route for some storage proteins. Bechtel et al (1982a), based on thin-sectioned material, concluded that the Golgi apparatus probably packages a small amount of the protein which accumulates in the endosperm. The large number of Golgi bodies depicted by high-voltage election microscopy (Parker and Hawes 1982) and now by freeze-fracture suggests that the Golgi apparatus plays a much more important role in storage protein secretion than we previously thought.

Bechtel et al (1982a,b) described pinocytotic vesicles associated with protein body membranes and the tonoplast. Our freeze-fracture study of freshly frozen and glycerol-imbibed endosperm samples exhibited the same vesicles, which correspond closely to pits and bulges observed in freeze-fracture micrographs showing endocytosis in animal cells (Orci and Perrelet 1975). The pinocytotic vesicles in wheat were thought to function as a mechanism of protein body enlargement, which suggested a soluble mode of protein secretion (Bechtel et al 1982a). With the establishment of ER-Golgi body connections and ER vesicle production, it now seems unlikely that these structures function in this manner. A more likely function is one involving the recycling or sequestering of membrane. Therefore, the vesicles and lamellar bodies found in vacuoles may represent protein body and vacuole membranes no longer needed. The starchy endosperm cells are dead at maturity, therefore there is no need to recycle membrane until germination. Possibly these membranous structures and other lipid-containing inclusions (Parker 1980) provide a lipid reserve for use during germination.

Our understanding of cereal endosperm development is far from complete. We have only just begun to unravel the complexity of how storage proteins are transported from the RER into vacuoles where they eventually form the matrix protein. Freeze-fracture provides a technique to study endosperm development without many of the problems associated with fixed tissues. In addition, it provides unique three-dimensional views of membranes and cytoplasm that are not available with thin-sectioned endosperm.

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