Protein Secretion in Wheat Endosperm—Formation of the Matrix Protein[1]

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ABSTRACT

The deposition of protein into vacuoles in the starchy endosperm of hard red winter wheat was studied using transmission electron microscopy and enzymatic digestion of thin sections. Protein bodies that formed in the cytoplasm were transported to the central vacuole where the protein body membrane and tonoplast fused and deposited the granule of protein into the vacuole. The protein granules in the vacuole enlarged by three mechanisms: addition of membranous vesicular material of various types; addition of flocculent material; and fusion of the granules with other newly deposited protein granules. The fusion process occurred rapidly after 17 days after flowering and resulted in the conversion of the spherical protein granules into irregularly shaped protein masses that eventually became the matrix protein. Enzymatic digestion of thin sections revealed that the contents of dense-cored Golgi vesicles and protein bodies were susceptible to protease VI and pepsin but not to α-amylase. The vacuolar protein granules were almost completely digested with protease VI and pepsin. The only undigested regions were peripheral densely stained inclusions thought to be the last added protein.

The cereal endosperm can be divided into three major morphological regions. The outermost region, the aleurone, is the only living endosperm tissue at maturity and forms a layer that is from one to several cells thick. Below the aleurone is the starchy endosperm, which is divided into the subaleurone region and central endosperm. The subaleurone is located just beneath the aleurone and is typified by a few small starch granules and large quantities of protein (Bechtel and Pomeranz 1978). The rest of the endosperm, the central endosperm, is typified by large numbers of large starch granules and small amounts of protein. The huge stores of carbohydrate and protein make cereals the world's major food supply. Wheat endosperm possesses an added feature: the milled endosperm can be converted into dough to yield a leavened product. This inherent ability resides primarily in the starchy endosperm storage proteins. Consequently, wheat endosperm development and, in particular, the formation of storage proteins have been intensively studied (Bechtel et al 1982). Most researchers agree that most of the wheat endosperm storage protein is secreted into vacuoles, except during late stages of development (Simmonds and O'Brien 1981).

Earlier we described wheat protein body initiation and enlargement and implicated the Golgi apparatus in the initiation of protein bodies (Bechtel et al 1982). We also showed that Golgi vesicles from a variety of cereal endosperms contained protease-digestible material (Bechtel and Gaines 1982). The work reported here describes the transport of protein bodies from their site of synthesis to deposition into the central vacuoles and depicts how the storage proteins in the vacuoles enlarge to eventually form the matrix in mature wheat endosperm.

MATERIALS AND METHODS

Hard red winter wheat cultivar Eagle was grown on outdoor plots and prepared for electron microscopy as previously described (Bechtel and Gaines 1982). Wet (as-is) kernel weights were determined by taking 10 kernels at each sampling date and weighing in a closed container. The same 10 kernels were dried at 110°C to constant weight to determine the kernel dry weight. Micronitrogen determinations were conducted by AOAC methods (1970).

Wheat caryopses used for enzymatic digestions were fixed as for routine microscopy or in 3% paraformaldehyde and 3% glutaraldehyde in 0.05*M* phosphate buffer without postfixation in osmium tetroxide (Bechtel and Gaines 1982). The unosmicated tissue yielded more consistent enzyme extractions than did the postfixed endosperm; however, tissue treated only in aldehyde was not as well fixed as the osmicated endosperm. The samples for enzyme digestion were further processed according to the schedule used in routine microscopy. Thin sections were cut consecutively and mounted singly on grids so that the effects of the various treatments could be followed for cellular components in the same cell. The enzymes are described and the digestions were conducted as previously outlined (Bechtel and Pomeranz 1981).

RESULTS

General

Figure 1 shows the relationships among kernel dry weight, water content, timing of protein body initiation, and protein per kernel. The dry weight of the kernel increased consistently during development, whereas the moisture content remained somewhat constant during the first 12 days and then dropped rapidly. Protein content per kernel increased consistently during caryopsis development and closely paralleled the dry weight data.

Protein Accumulation

Because of the complexity by which storage protein is secreted in wheat, several clarifying definitions are required. In this article, a protein body is defined as a discrete mass (granule) of storage protein surrounded by a single trilaminar membrane. A protein

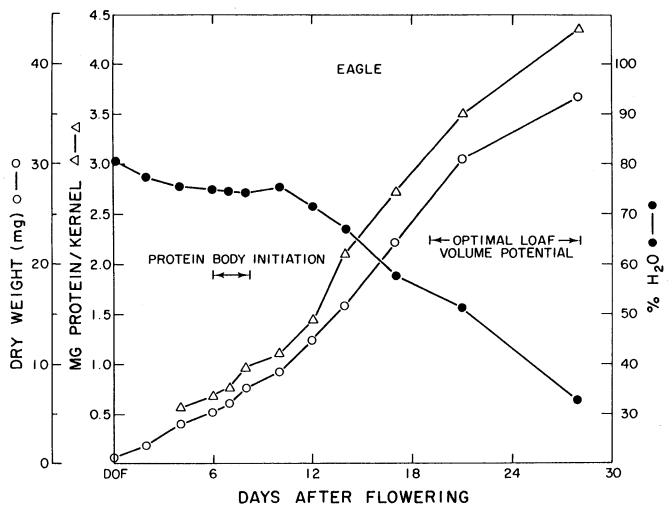

Fig. 1. Dry weight, moisture content, and protein content of hard red winter wheat Eagle during caryopsis development.

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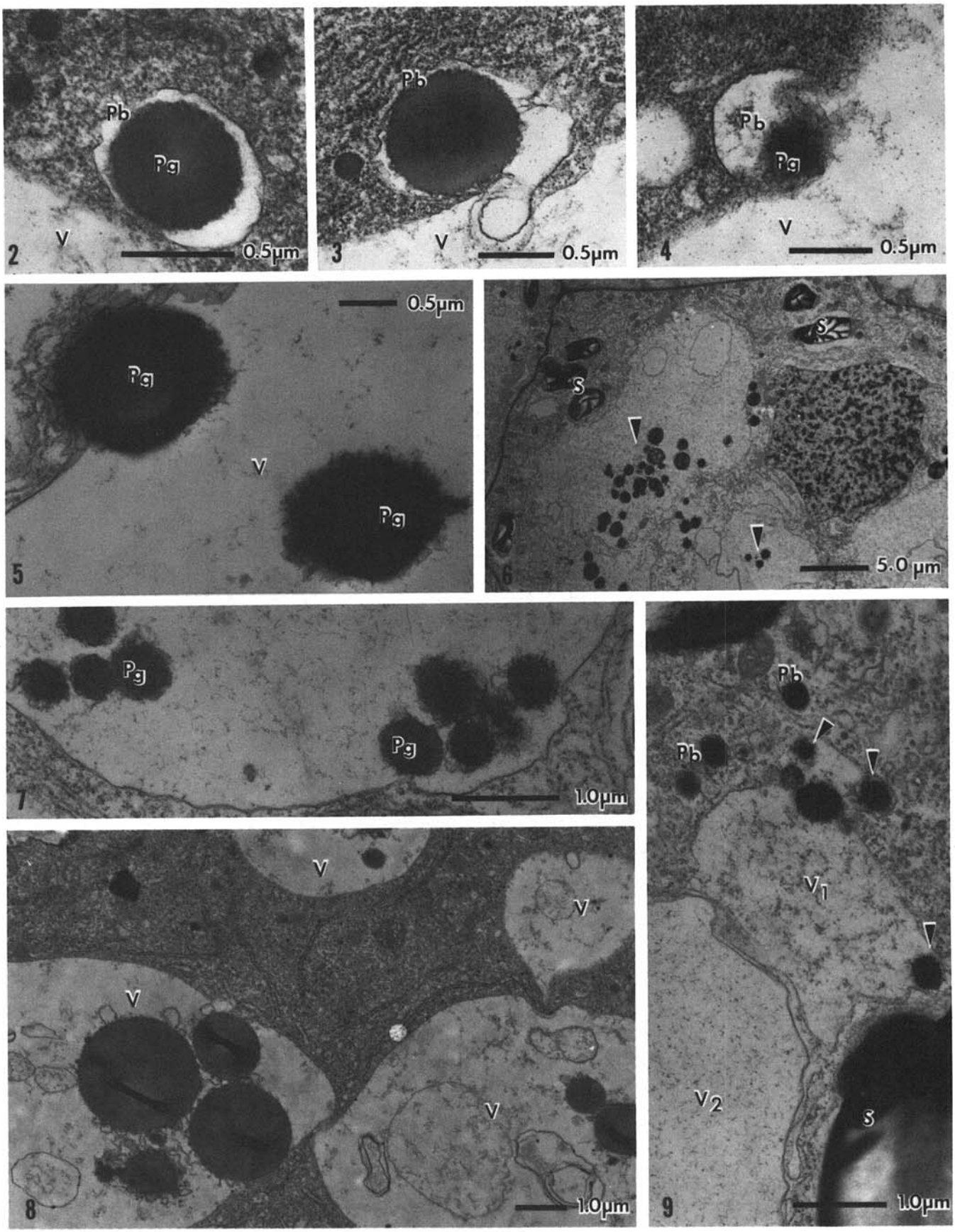


Fig. 2. Protein body (Pb) with protein granule (Pg) associated with vacuole (V) at 10 days after flowering (DAF) (×42,600). **Fig. 3.** Membrane of protein body (Pb) and vacuole (V) fusing at 10 DAF (×27,300). **Fig. 4.** One section of a series of serial sections showing a 10-DAF protein granule (Pg) of protein body (Pb) entering vacuole (V) following membrane fusion (×31,300). **Fig. 5.** Two protein granules (Pg) in vacuole (V) at 7 DAF (×21,600). **Fig. 6.** Numerous protein granules in vacuoles (arrows) at 7 DAF. S = starch granules (×2,500). **Fig. 7.** Protein granules (Pg) in central vacuole at 7 DAF (×20,000). **Fig. 8.** Four vacuoles (V) in center of endosperm cell at 10 DAF. Some of the vacuoles have protein granules (×9,600). **Fig. 9.** Protein body (Pb) near vacuole (V_1) at 7 DAF in which protein granules have just been deposited (arrows). Large vacuole (V_2) and smaller one (V_1) are about to fuse near starch granule (S) (×18,000).

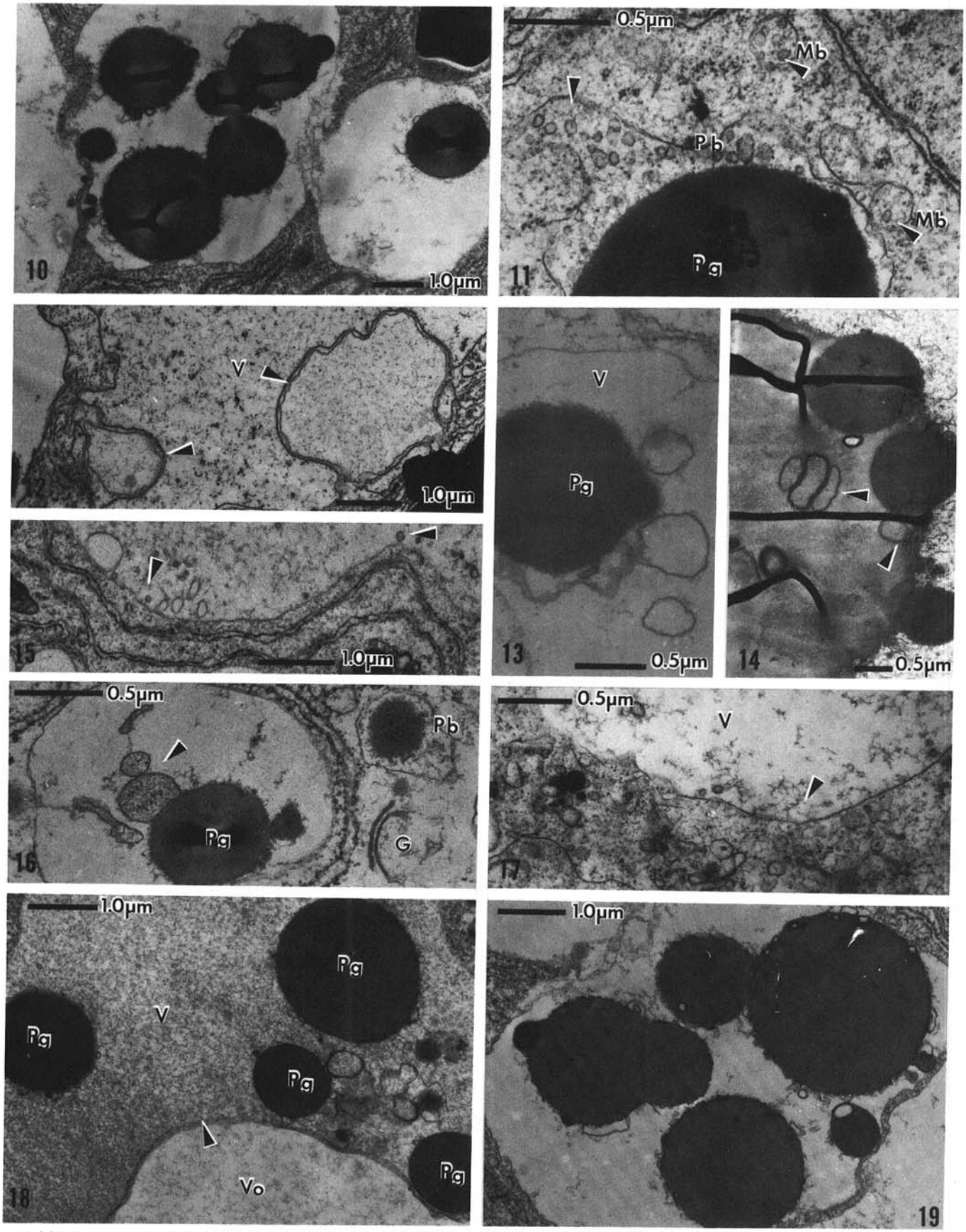
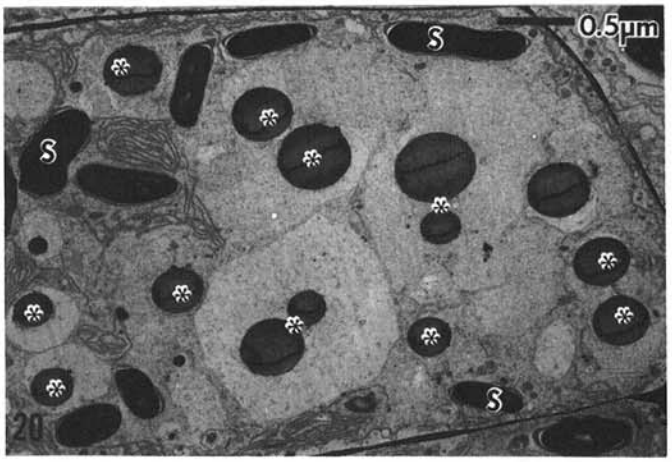
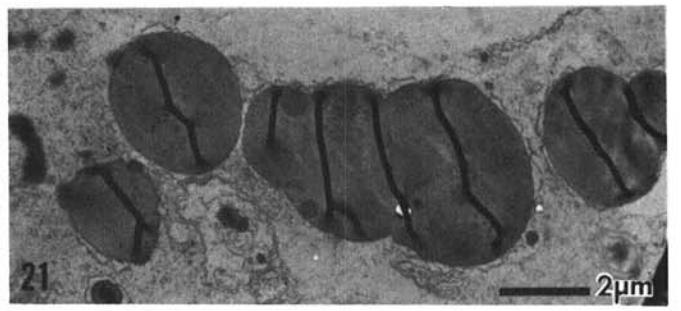
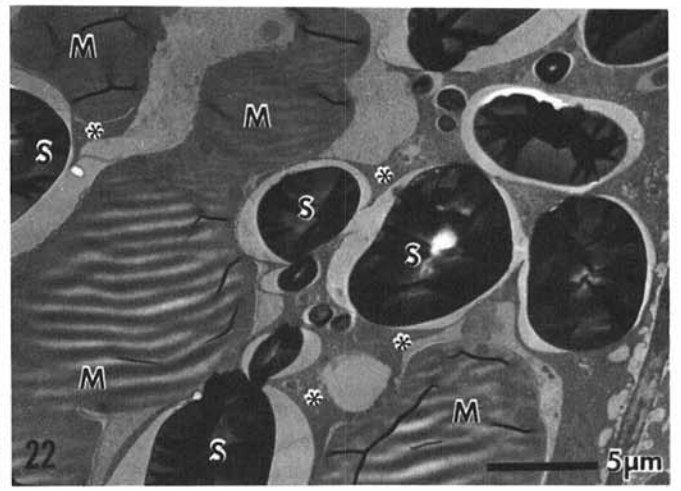
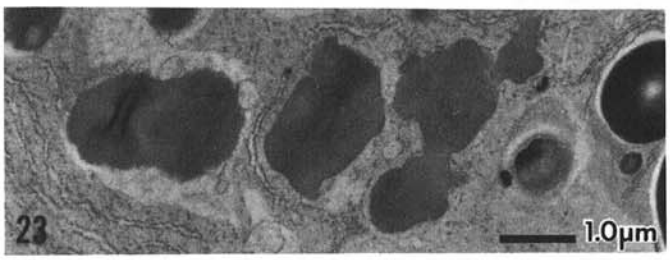


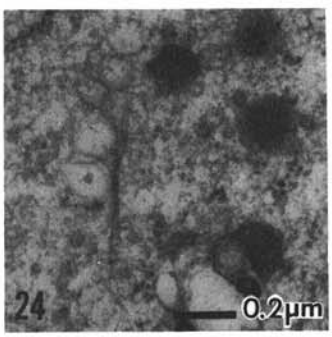
Fig. 10. Membranes between three adjacent vacuoles beginning to fuse at 10 days after flowering (DAF). Note the typical characteristic of section folding in the protein granules (×9,400). **Fig. 11.** Multivesicular bodies (Mb) containing small vesicles at 12 DAF near protein body (Pb). Note similar vesicles surrounding protein granule (Pg) and apparent fusion of multivesicular body with protein body (arrow) (×37,000). **Fig. 12.** Large vesicle (arrow) blebbing from tonoplast into vacuole (V) at 7 DAF (×12,400). **Fig. 13.** Vesicles associated with protein granule (Pg) in vacuole (V) at 14 DAF (×26,100). **Fig. 14.** Vesicular material incorporated into protein granule (arrows) at 12 DAF (×13,900). **Fig. 15.** Small vesicles (arrows) pinching off of tonoplast at 12 DAF (×14,000). **Fig. 16.** Vesicles of cytoplasm (arrows) associated with protein granule (Pg) at 12 DAF. Pb = protein body, G = Golgi body (×32,100). **Fig. 17.** Flocculent material (arrow) in vacuole (V) at 12 DAF (×28,200). **Fig. 18.** Protein granules (Pg) in dense flocculent material at 10 DAF. Vacuole (V) has void (Vo), but void has no trilaminar membrane (arrow) (×12,800). **Fig. 19.** Fusion of protein granules in a vacuole at 10 DAF (×12,700).

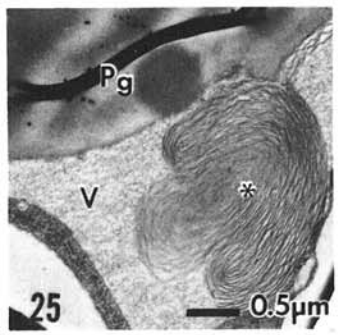


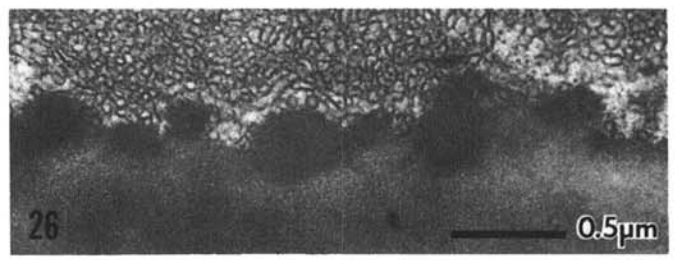


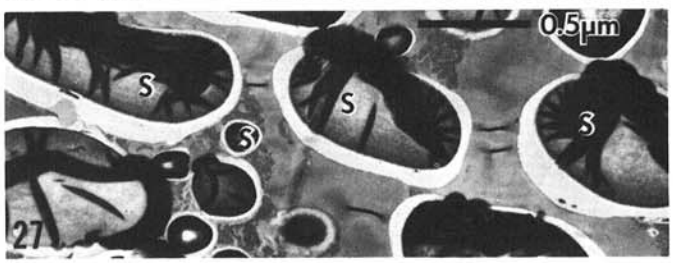


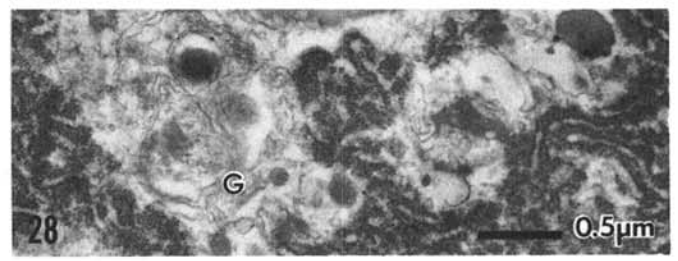


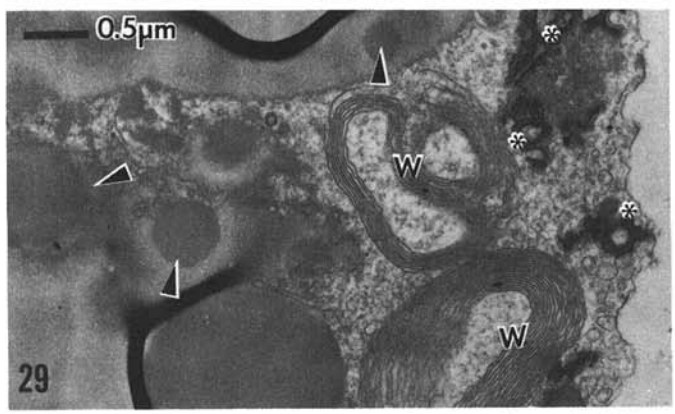












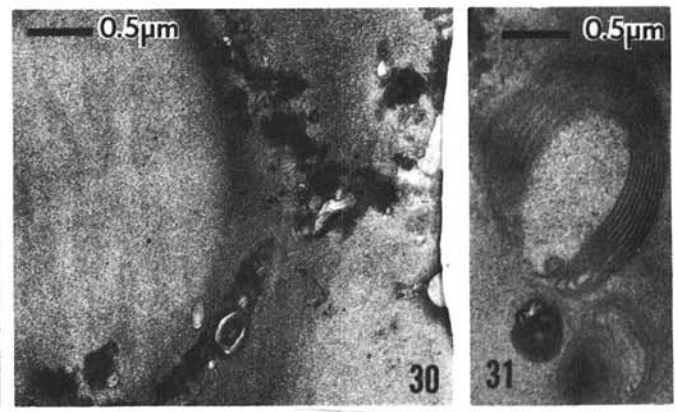


Fig. 20. Numerous individual protein granules (*) and starch granules (S) in endosperm at 14 days after flowering (DAF) (×2,000). **Fig. 21.** Protein granules fusing at 17 DAF (×6,050). **Fig. 22.** Wedge-shaped cytoplasm (*) between developing protein matrix (M) and starch granules (S) at 21 DAF (×2,900). **Fig. 23.** Small protein granules fusing at 21 DAF (×10,000). **Fig. 24.** Golgi body with dense vesicles in cytoplasm at 21 DAF (×39,000). **Fig. 25.** Large lamellar structure (*) in vacuole (V) associated with protein granule (Pg) at 21 DAF (×14,300). **Fig. 26.** Netlike mass around portion of developing matrix protein at 21 DAF (×29,000). **Fig. 27.** Endosperm of 28-DAF caryopsis with large and small starch granules (S) embedded in protein matrix (×3,000). **Fig. 28.** Golgi apparatus (G) in endosperm producing dense vesicles at 28 DAF. Note large amounts of rough endoplasmic reticulum (×22,400). **Fig. 29.** Distended ER (*) in cytoplasm at 28 DAF. Note lamellar whorls (W) and dense inclusions (arrows) in matrix protein (×16,600). **Fig. 30.** Dense inclusions in protein matrix at 28 DAF (×17,300). **Fig. 31.** Lamellar material in protein matrix along with dense inclusions at 28 DAF. (×18,400)

body may have a single granule (Figs. 2–4) or multiple granules (a protein mass) within the enclosing membrane (Figs. 5–7). Therefore, many protein granules may be present within a large vacuole (protein body), or the granule may occur as a single vesicle in the cytoplasm (also a protein body).

After protein bodies were initiated, they were transported through the cytoplasm for deposition into the large central vacuoles. Enlargement of the protein body occurred during the movement from the Golgi apparatus to the vacuoles (Bechtel et al 1982). The protein body and vacuole membranes fused when the protein body reached the vacuole surface (Figs. 2–4) and deposited the protein granule into the vacuole (Fig. 5). The result at seven days after flowering (DAF) was several protein granules within the central vacuoles (Figs. 6 and 7). In many cases, particularly in the subaleurone cells, the center of the cell was not composed of a single large central vacuole but had instead several smaller vacuoles (Fig. 8). These smaller vacuoles fused with one another to eventually form the large central vacuole (Figs. 9 and 10).

Three mechanisms were observed by which protein granules within the central vacuole and surrounding smaller vacuoles enlarged. The first mechanism was the addition of three types of membranous material to the protein granules. Small vesicles within multivesicular bodies were added to protein granules when the protein body and multivesicular body membranes fused (Fig. 11). Large membranous vesicles from the tonoplast blebbed out into the vacuole, pinched off, became associated with the protein granules (Figs. 12 and 13), and eventually became incorporated into the granules (Fig. 14). Pinocytotic vesicles (Fig. 15), some of which contained substantial amounts of cytoplasm (Fig. 16), were present within vacuoles that contained protein granules. A second consistently observed phenomenon was the presence of flocculent material in the large vacuoles during protein body enlargement. This material was first associated with the tonoplast and projected into the vacuole (Fig. 17). Much of the time, the flocculent material was relatively dispersed (Fig. 17), but occasionally it became extremely dense (Fig. 18). This material was persistent throughout protein deposition into the large central vacuoles. The third mechanism of protein body enlargement within the central vacuoles was the fusion of the small individual granules among one another to form even larger granules (Fig. 19). The appearance of the protein granules at 14 DAF was represented as individual large spherical structures about 4 μm in diameter (Fig. 20).

Matrix Formation

At 17 DAF, a change was observed in the protein granules. Instead of the large granules remaining individual, they began to fuse with one another (Fig. 21). This fusion process progressed rapidly, and by 21 DAF most of the vacuolar granules (Fig. 22) and many of the small granules in vesicles (Fig. 23) had at least partially fused with one another into irregularly shaped protein masses. The enlarging protein masses in the vacuoles were then partially converted into matrix protein when the starch granules pushed into the vacuole and deformed it. The growth of starch granules and addition of storage protein resulted in forcing the cytoplasm into wedge-shaped regions (Fig. 22). Even at this late stage of development, as in the earlier stages, the Golgi apparatus was conspicuously producing electron-dense vesicles (Fig. 24) within the increasingly dense cytoplasm (Figs. 23 and 24). Large lamellar structures (Fig. 25) and netlike masses (Fig. 26) were observed in vacuoles for the first time at 21 DAF.

The protein matrix had proliferated to completely surround the plastids by 28 DAF. The proliferation isolated many of the wedges of cytoplasm between plastids (containing large starch granules) and protein matrix (Fig. 27). What seemed to be Golgi apparatus was still present in the very dense cytoplasm and produced electron-dense vesicles (Fig. 28). Rough endoplasmic reticulum (ER) had become distended, and the lumen was filled with flocculent material (Fig. 29). Lamellar whorls were much more common at 28 DAF (Fig. 29) than at the earlier stages. The protein matrix was relatively homogeneous but was interrupted by spherical electron-dense inclusions at the protein matrix periphery (Fig. 29). When the protein matrix from two different areas were pushed together and fused, irregular dense inclusions became embedded in the matrix (Figs. 30 and 31). The cytoplasm was extremely dense at 28 DAF and consisted primarily of polysomes and rough ER (Fig. 32).

The wheat was mature and ready for harvesting at 34 DAF. The remaining cytoplasm consisted of rough ER and ribosomes compressed into small areas (Fig. 33). The protein matrix at 34 DAF (Fig. 34) was indistinguishable from that of 28 DAF (Fig. 30) and contained the same inclusions as the 28 DAF wheat.

Enzymatic Digestions

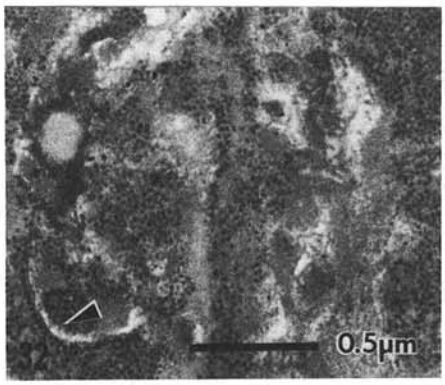
Enzymatic digestion of thin sections was conducted to determine the location of storage protein. Both protease VI and pepsin digested the densely stained Golgi vesicles (Figs. 35a,b; 36a,b), whereas α-amylase did not digest the Golgi vesicle contents (Fig. 37a,b). Digestion of the vacuolar protein granules with protease VI and pepsin resulted in nearly complete removal of the matrix and very little removal of the densely stained peripheral inclusions (Fig. 38a,b,c). Pepsin digestion showed a gradation in resistance of the inclusion to digestion for some of the inclusions (Fig. 38c). An extensive search was made to locate cytoplasmic vesicles that possessed protease resistance and could be related to the dense inclusions in the matrix protein. None could be found in any sample (Figs. 39a,b; 40a,b). The only time that resistant granules were observed was after they were located inside the vacuole and in close proximity to the protein matrix (Fig. 40a,b). The only structures in the cytoplasm that resembled the dense inclusions were lipid droplets, and these structures were not present in large quantities, especially in the central endosperm region.

DISCUSSION

Wheat protein is unique among cereals (except for Triticale) in that the starchy endosperm can be readily converted into a dough possessing rheological properties suitable for making leavened products. The protein matrix of the wheat endosperm suggests a reason for its unique properties. Many other cereals that lack wheat's dough-producing capability have individual protein bodies rather than a protein matrix at maturity. These cereals include rice (Bechtel and Juliano 1980, Bechtel and Pomeranz 1978), oats (Bechtel and Pomeranz 1981), sorghum (Adams and Novellie 1975, Seckinger and Wolf 1973), proso millet (Jones et al 1970), finger millet (Adams and Liebenberg 1975), corn (Adams et al 1976, Khoo and Wolf 1970), and pearl millet (Adams et al 1976). However, a few cereals such as barley (Bechtel and Pomeranz 1979) and rye[4] also possess a protein matrix at maturity. Under appropriate conditions, the proteins of these two cereals can be extracted and the extracted proteins possess viscoelastic properties (Cunningham

[4] Bechtel and Gaines. Unpublished data.

Fig. 32. Numerous polysomes and some rough endoplasmic reticulum (arrow) at 28 days after flowering (DAF) (×34,500). **Fig. 33.** Rough endoplasmic reticulum and polysomes in mature endosperm at 34 DAF (×39,500). **Fig. 34.** Protein matrix of mature endosperm at 34 DAF (×39,600). **Fig. 35. a,** Golgi body and vesicles in control for protease VI digestion of endosperm at 12 DAF (×28,100); **b,** Protease VI digestion of Golgi vesicles (×41,400). **Fig. 36. a,** Golgi body and vesicles in unstained control section for pepsin digestion at 14 DAF; **b,** Pepsin digestion of same Golgi vesicles in next serial section of (a) (×46,000). **Fig. 37. a,** Golgi body and vesicles in control section for α-amylase digestion at 12 DAF; **b,** Golgi body after α-amylase digestion. Note lack of digestion (×56,400). **Fig. 38 a,** Protein body in control section for pepsin digestion at 12 DAF. Note dense inclusions between protein granules (×7,500); **b,** Protease VI digestion removed matrix (*) but left inclusions intact (×39,100); **c,** Pepsin digestion removed most of matrix protein (*) but left most inclusions intact. Note that one inclusion (arrow) was partially digested (×18,300). **Fig. 39. a,** Control for pepsin digestion in endosperm at 14 DAF; **b,** Pepsin digestion of next serial section showing digestion of same protein bodies (×12,500). **Fig. 40. a,** Control of pepsin digestion in endosperm at 21 DAF; **b,** Pepsin digestion of next serial section showing removal of protein matrix (*) and of protein bodies (arrows) but not dense inclusions (I) (×14,100).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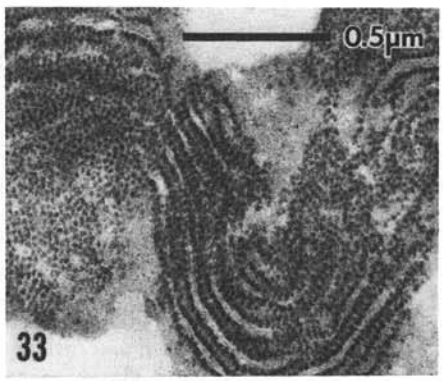
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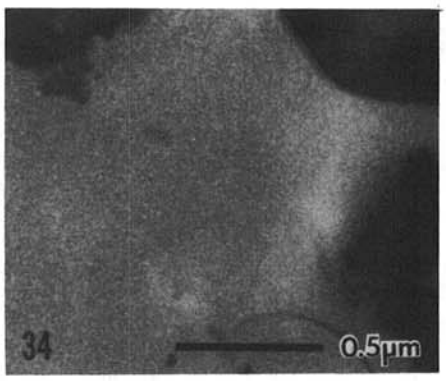
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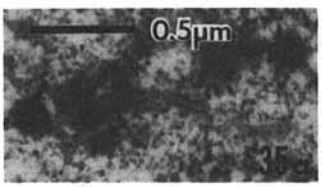
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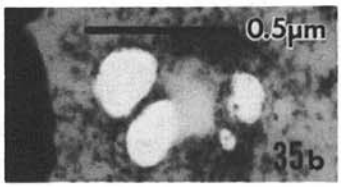
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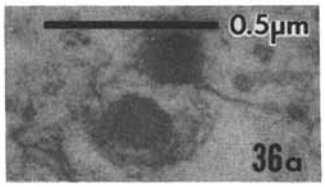
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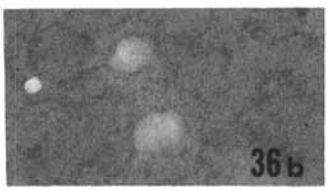
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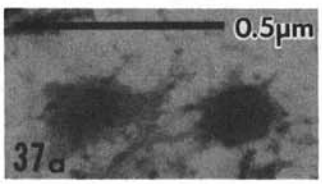
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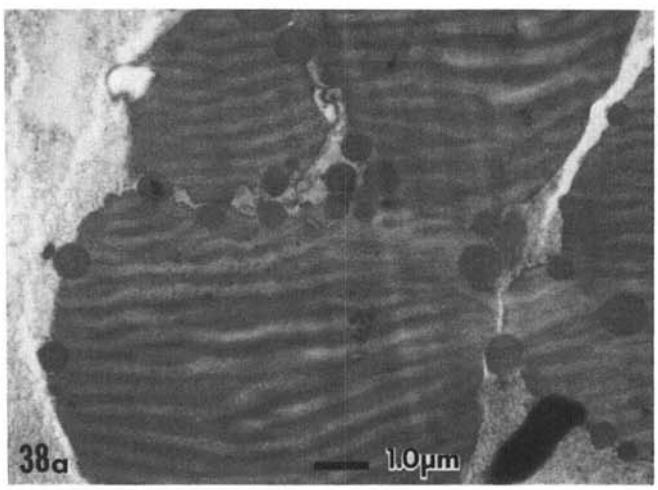
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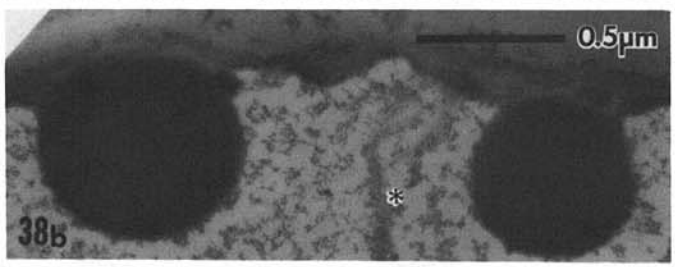
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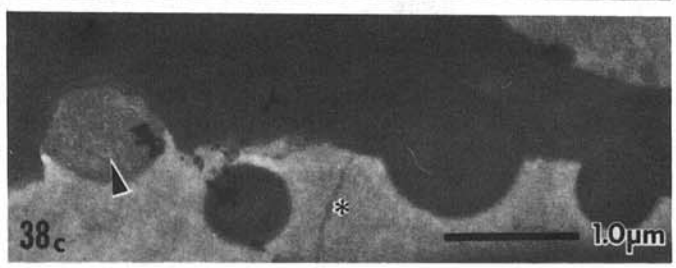
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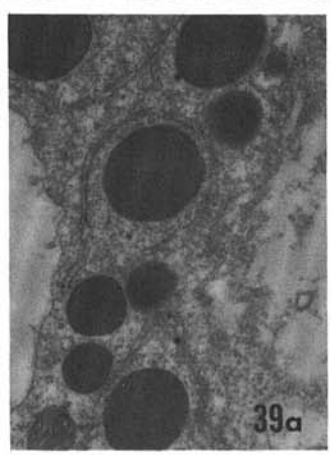
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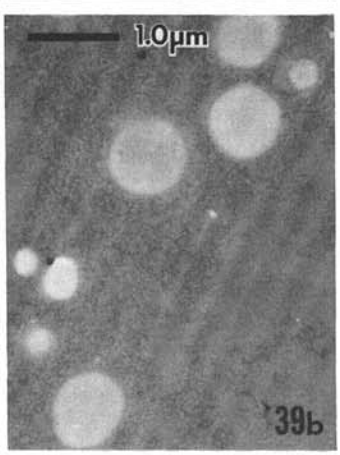
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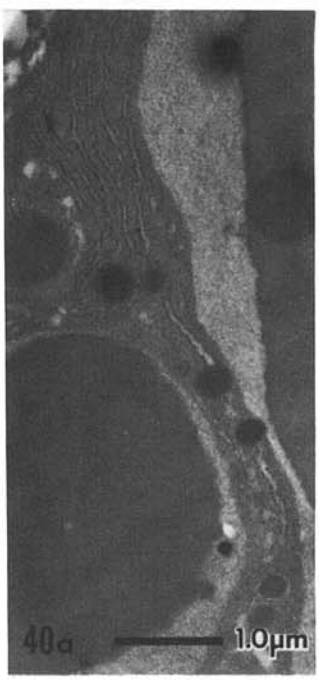
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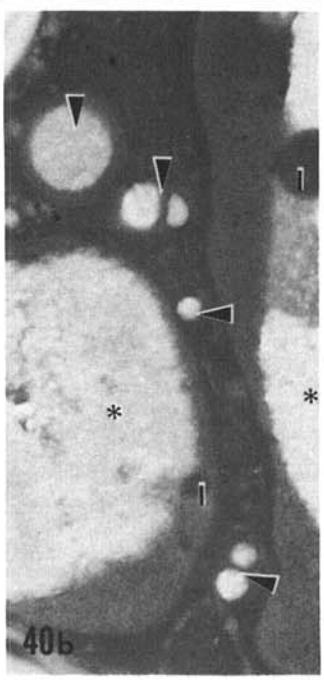
40b

et al 1955a, 1955b). Consequently, the matrix appearance of mature wheat and other cereal endosperm proteins may be indicative of potential breadmaking qualities.

The fact that wheat endosperm does form a protein matrix during development is probably one reason for so many varied theories on the mechanism of protein accumulation. This is primarily because the matrix complicates interpretations. The proteoplast theory of protein body formation in wheat (Morton, Palk and Raison 1964, Morton and Raison 1963) has not been substantiated (Bechtel et al 1982, Campbell et al 1981, Simmonds and O'Brien 1981). The accumulation of protein in wheat in vacuoles has been well documented (Barlow et al 1974, Briarty et al 1979, Buttrose 1963, Campbell et al 1974, Graham et al 1962, Harvey et al 1974, Parker 1980, Simmonds 1978); however, the mechanisms by which the protein entered the vacuoles was not understood (Campbell et al 1981, Simmonds and O'Brien 1981).

Our work on the initiation of protein bodies (Fig. 41) indicated that dense-cored Golgi-derived vesicles were the initiation sites of protein bodies (Bechtel et al 1982). These vesicles became protein bodies when they enlarged via three mechanisms. Furthermore, we have shown that the Golgi vesicle contents were protease-digestible (Bechtel and Gaines 1982). The results of the present study have revealed that the protein bodies located in the cytoplasm were transported to the central vacuoles and fused with the vacuoles to become the vacuolar protein. At no time during the early stages of protein body development did continuities appear between rough ER and protein bodies (Bechtel et al 1982). The mechanisms by which vacuolar protein deposits enlarged were similar to those that enlarged Golgi vesicles into protein bodies. The major difference was that much more fusion of deposits occurred in vacuoles than in the smaller protein bodies.

The fusion process increased noticeably during the 14–17 DAF period and peaked at the 21–28 DAF period. Finney (1954) found the wheats harvested 10–14 days preripe (20–28 DAF by our calculations) had maximum loaf volume potentialities and superior physical flour properites than the same wheat harvested at other times, including harvest maturity (Fig. 1). The loaf volume potential rose rapidly after 20 days preripe (16 DAF). Loaf volume potential typically resides with the gliadin fraction of wheat flour proteins (Hoseney et al 1969). Recent work by Mecham et al (1981) has shown that the α, β, and some γ-gliadins were synthesized rapidly between 12 and 18 DAF. Indeed, the greatest jump in concentration of higher molecular weight gliadins (~36,000 da) occurred at 18 DAF. Consequently, good indirect evidence indicates that increased protein body fusion up to a certain level is concomitant with increases in both loaf volume potential and gliadin synthesis.

An interesting phenomenon observed from seven DAF up to and including maturity was the electron-dense vesicular-shaped material at the protein body periphery. This material has often been observed, and a variety of origins and functions were ascribed to the inclusions (Parker 1980). The inclusions' dense staining and lack of susceptibility to proteases (Figs. 37a–c, 38a,b; Parker 1980) suggest that they may have a different composition than the rest of the protein aggregate. We made an intensive search of our pepsin and protease VI-digested sections to locate similarly stained deposits in the cytoplasm. The only structures of similar appearance that were not digested were the numerous lipid droplets of the aleurone layer, the few lipid droplets of subaleurone, and very rare droplets of the central endosperm. No protease resistant structures surrounded by a distinct membrane were observed within the cytoplasm. The dense inclusions were almost always at the periphery of the protein mass except when the protein masses fused, and the dense inclusions at the junction were incorporated (Fig. 38a), or when an oblique section through the edge of a protein mass revealed the inclusions to be in the center. All those observations would suggest the possibility that they were deposited at one time and "float" to the surface of the protein mass. This hypothesis can be ruled out, however, for several reasons: the inclusions increase in number throughout protein deposition; the electron density is variable—from that of the protein matrix to that of the densest; some of the inclusions exhibit a gradation of susceptibility to proteases (Fig. 38c); and the edge of the dense inclusion often blends into the protein mass (Figs. 10, 19, 25, 26). For these reasons, we have suggested that the dense inclusions are the last added protein. If this were the case, an explanation would be needed to explain the varied extraction of proteins by proteases. No present data support a theory involving protein modification that makes the proteins resistant to proteases. Parker (1980), however, has used histochemical techniques to show that the dense inclusions contained lipoproteins. This composition might explain the resistance of the dense inclusions to protease attack and the gradual transition of the inclusions into matrix protein. A phenomenon possibly related to this problem was that observed by Mecham et al (1981) in which the gliadin proteins of developing wheat were poorly extracted by aluminum lactate buffer, whereas mature proteins were easily extracted. They suggested that aluminum lactate buffer at pH 3 may not remove all the gliadins because these proteins may be in a configuration that is stable at pH 3, whereas the use of other extracts at other pHs would allow for better removal of the gliadins.

The formation of membranous and lamellar inclusions in the endosperm vacuoles suggests fixation artifacts caused by the tissue preparation. We feel that this is not the case with our material because the tonoplast and other membranes of the cytoplasm are well preserved and continuous. In addition, these structures are observed within the protein matrix in both developing and mature wheat endosperm. These observations plus the results of Parker (1980) suggest that these structures are real. Parker (1980) found that the inclusions were present regardless of the fixation procedure. Our hypothesis on the inclusions is that they may represent extra membrane produced by the numerous vesicle-vacuole fusions that have occurred throughout protein deposition. They form primarily as a way to rid the tonoplast of extra membrane, and because the starchy endosperm is dying, this mechanism allows for the removal of the membrane without the normal recycling procedure.

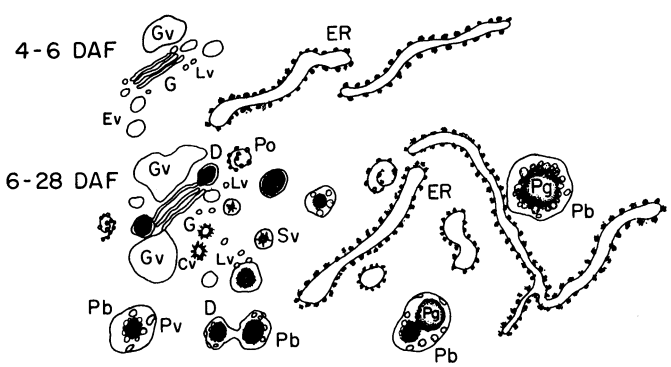

Fig. 41. Diagrammatic scheme of protein body initiation in wheat starchy endosperm. Before protein bodies were initiated (4–6 days after flowering) (DAF), the Golgi apparatus (G) secreted electron-lucent vesicles (Ev), small lucent vesicles (Lv), and Golgi vacuoles (Gv). After protein bodies were initiated (6 DAF) and throughout protein body formation (6–28 DAF), the Golgi apparatus secreted vesicles with star-shaped inclusions (Sv) and dense-cored vesicles (D) in addition to the vesicles secreted prior to protein body initiation. Rough endoplasmic reticulum (ER) was present throughout endosperm development. Polysomes (Po) associated with the Golgi apparatus and ER. Pinocytotic vesicles (Pv) were associated with protein bodies (Pb) frequently, whereas lucent vesicles (Lv) and dense-cored vesicles (D) were not. Protein bodies (Pb) with protein granules (Pg) often fused with one another to form large protein bodies.

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