

# The Ultrastructure of the Mature Wheat Endosperm<sup>1</sup>

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## ABSTRACT

The storage protein of the wheat endosperm may be separated from the starch component under nonaqueous conditions involving air classification followed by flotation in mixtures of chloroform and benzene of density 1.32 to 1.34. The small pieces of protein thus obtained, together with coarsely milled fragments of the original endosperm, were examined by electron microscopy. Numerous osmiophilic inclusions were observed in each preparation. Those inclusions showing residual structure were identified as remnants of the amyloplast membranes surrounding each starch granule and of the endoplasmic reticulum of the developing endosperm cell. Ribosomes could be seen attached to the latter. Sections of hard and soft wheat differed in appearance under the electron microscope, and this appeared to be related to differences in the extent of shrinkage of the protein matrix during fixation, and in the strength of adhesion between starch and protein in the two wheat types.

Because of its effect on the interrelationship of the starch and protein components, the ultrastructure of the wheat endosperm plays a major role in determining the milling behavior of the grain and the rheological properties and baking performance of wheat-flour doughs. Comprehensive studies of endosperm ultrastructure in developing wheat were carried out by Buttrose (1,2) and by Morton and co-workers (3,4). Because of technical difficulties, these workers did not examine by electron microscopy any material more mature than 25 days after anthesis.

Hess and Mahl (5) used replica coating techniques to investigate flour protein and starch fractions which had been separated by flotation and sedimentation in organic solvent mixtures (6,7). Hess concluded that the endosperm protein consisted of two components, one of which adheres closely to the surface of the starch granules, while the other fills the wedge-like spaces between the granules (8). The adherent protein had an average thickness of 0.217  $\mu\text{m}$ . and could be seen only under an electron microscope.

Although more recent work has been carried out on the separation of starch and protein from pin-milled flour (9,10,11), the difficulties referred to above have, until recently, prevented the sectioning of mature wheat grains and the examination, *in situ*, of the starch and protein components.

However, in 1967 Seckinger and Wolf (12), using "Maraglas" epoxy resin as an embedding agent, examined the lipid distribution in the mature wheat endosperm, demonstrating the appearance of osmiophilic inclusions in the protein matrix which they believed to be remnants of plastid membranes and cytoplasmic bodies present at the time of kernel maturation. They were unable to confirm the differentiation into wedge and adherent endosperm protein proposed by Hess, nor did they consider that there was evidence for a continuous layer of protein surrounding each starch granule in the form of a membrane.

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In an attempt to resolve the conflicting data presented in the literature, and to determine the nature of the membranous residues in the mature endosperm cell, with particular reference to the survival of the amyloplast membrane, we have applied the newer embedding techniques of Luft (13), Feder and O'Brien (14), and Spurr (15) to whole grain and to certain of its milled and fractionated components.

## MATERIALS AND METHODS

### Preparation of Air-Classified Flour Fractions

Straight-run flours were prepared by Buhler milling from the hard white spring wheat varieties Timgalen and Gamenya, and from the soft white spring varieties Summit, Olympic, and Heron. These were pin-milled on a MiniKEK mill at a feed rate of approximately 40 lb. per hr. and a rotor speed of 12,000 r.p.m. Examination of samples under the microscope showed considerable release of starch granules after pin-milling.

The pin-milled samples were air-classified on a Miag air classifier with a throughput of 1,000 to 1,800 kg. of flour per hr. By disconnecting the infeed hopper and the continuous valve system it was possible to run 40 to 60 lb. of pin-milled flour through in approximately 5 to 10 min. and to collect the air-classified fractions directly from the appropriate cyclone. The flour was thus subdivided into three portions: protein-rich (0 to 17  $\mu\text{m}$ .), starch-rich (17 to 35  $\mu\text{m}$ .), and overtailings ( $>35$   $\mu\text{m}$ .). The size ranges were checked by microscopic observation.

### Preparation of Purified Storage Protein

The fine fraction was used for the preparation of purified storage protein by flotation in chloroform-benzene mixtures of densities ranging between 1.32 and 1.34, using a modification of the methods of Hess (9), Stevens et al. (10), and Kent and Evers (11). Highly purified storage protein preparations could be obtained after two or three treatments by adjusting the density of the suspending solvent to 1.32 in later sedimentations. Settling under gravity, although slower than centrifugation, gave purer preparations with fewer steps.

It will be seen from Table I that the storage protein so obtained still contained some 4 to 9% impurity, consisting mainly of occluded small starch granules. To completely remove these it was necessary to ball-mill the protein after the second or third suspension, and subsequently subject it to a final resuspension and flotation.

The progress of purification was monitored by light microscopy and by nitrogen determination through the micro-Kjeldahl procedure. Dilute aqueous fast green (0.5%) was used to stain endosperm storage protein; iodine (0.2%) in potassium iodide (2%), for starch; and dilute aqueous toluidine blue (0.05%), for aleurone cell contents and for pericarp contamination. High-protein fractions very readily form gluten during their preparation for microscopy. This was avoided by first smearing the preparation on the slide dry, then adding a drop of stain without mixing, and applying the coverslip.

### Embedding and Microscopy

Endosperm pieces measuring approximately 1 mm.<sup>3</sup> were cut from mature

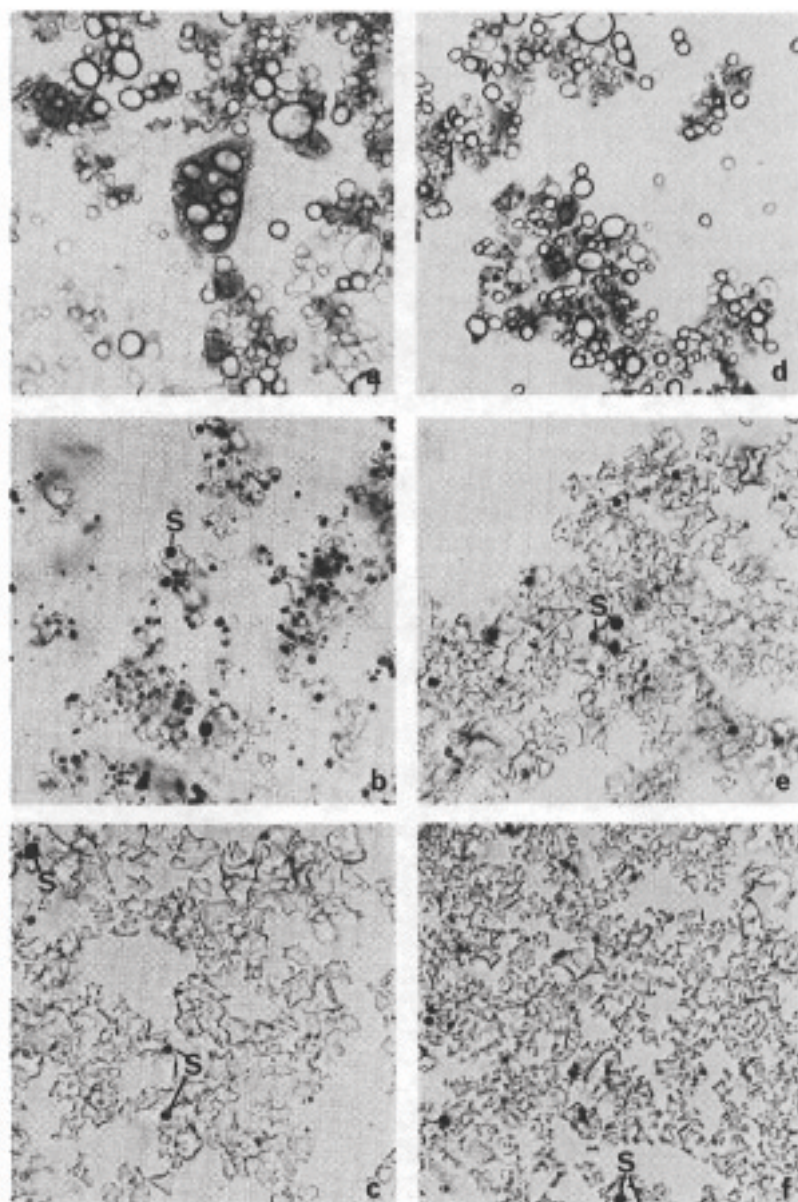


Fig. 1. Stages in purification of storage protein from Australian wheats. Parts a to c, hard white spring wheat, Timgalen; d to f, soft white spring wheat, cv. Heron; a and d, fine fraction (0 to 17  $\mu$ m.) as recovered from the air classifier, stained with fast green; b and e, storage protein after one purification step, stained with iodine; c and f, purified storage protein stained with iodine to show residual starch contamination. All magnifications 500X. Small contaminating starch granules are indicated by "S".

wheat grains with a razor blade. They were fixed in 10% acrolein in distilled water at 2°C., followed by dehydration, infiltration, and polymerization with glycol methacrylate—essentially according to Feder and O'Brien (14). The infiltration step was found to be critical; and the longer the period which could be allowed for this process (up to several weeks), the better were the final results. Sections were cut at 1.0  $\mu\text{m}$ . on a Cambridge microtome, Huxley pattern, and examined by light microscopy after appropriate staining, as described above.

Electron microscopy was carried out on endosperm pieces and on samples of semolinas derived from the coarse air-classified fractions obtained as described above. These were fixed in 3% glutaraldehyde in phosphate buffer (0.025M, pH 6.8) and 2% osmium tetroxide in the same buffer, embedded in low-viscosity epoxy resin (15) or Epon (13), and sectioned on an L.K.B. ultramicrotome. Sections 600 to 900 A thick were examined using a Phillips "300" electron microscope.

## RESULTS AND DISCUSSION

### Purification and Examination of Storage Protein

The appearance of separated wheat storage protein through successive stages of purification by resuspension in chloroform-benzene mixtures was examined by differential staining and light microscopy. Figure 1 illustrates the difference in appearance, at each stage, of fractions derived from a typical soft white Australian spring wheat (cv. Heron) and a hard spring wheat (cv. Timgalen).

Marked differences in the behavior of the hard and soft wheats during purification were observed. The soft wheats Heron and Olympic (particle size index [p.s.i.] (16) 30 and 29, respectively) fractured readily during pin-milling into discrete particles of storage protein and free starch granules. The hard wheat Timgalen (p.s.i. 17) yielded a storage-protein fraction which contained numerous small starch granules firmly attached to, or occluded by, the storage-protein particles. A wheat of intermediate hardness, Gamenya (p.s.i. 23), behaved more like the soft wheats in segregating cleanly during the sedimentation procedure. Yields of purified storage protein from Timgalen were therefore lower than those from the soft wheats, although by careful selection of the density of the solvent used for sedimentation, preparations of reasonable purity were obtainable.

Table I summarizes the protein contents of storage-protein preparations from two soft, one intermediate, and one hard white spring wheat at various stages of purification.

TABLE I. PROTEIN CONTENT OF STORAGE-PROTEIN PREPARATIONS FROM HARD AND SOFT WHEATS AT VARIOUS STAGES OF PURIFICATION

	Protein Content (N X 5.7) (percent dry weight)			
	Heron	Olympic	Gamenya	Timgalen
Parent flour	8.8	8.5	12.1	14.7
Air-classified fine fraction	17.4	18.1	19.4	20.0
Protein resuspended once	93.0	92.3	93.5	94.1
Protein resuspended twice	95.6	92.5	94.0	96.0
Gluten	86.0	85.8	84.7	85.2

After ball-milling and resedimentation in a chloroform-benzene mixture of density 1.32, storage-protein preparations of a purity exceeding 99% dry weight were obtained.

Starch-gel electrophoresis patterns of 2M urea extracts of the purified storage protein were compared with similar extracts from the original flour; from the protein-enriched fine fraction of the air-classified flour; and from the freeze-dried gluten. In each case, for a particular variety, the patterns were identical. They corresponded mainly to the bands given by the gliadin group of proteins having electrophoretic mobilities intermediate between those of the glutenin (slow-moving) and albumin-globulin groups (17). The storage-protein preparations contained a higher ratio of gliadin to albumin-globulin bands than the flour extracts.

Endosperm sections approximately 1 mm.<sup>3</sup> were embedded and examined by light microscopy. These indicated that the higher ratio of protein to starch in the subaleurone cells would make them more suitable for subsequent electron microscopy than pieces derived from the mid-endosperm regions of the grain.

Individual pieces of purified protein matrix were also embedded, sectioned, and examined by electron microscopy. The edge of each piece of matrix was outlined where it had abutted onto a starch granule, suggesting the presence of a membrane residue at this interface. A functional membrane, surrounding each amyloplast, would have existed at this point in the developing endosperm cell.

#### **Electron Microscopy of Wheat Semolinas and Cut Sections**

To determine to what extent these and other osmiophilic inclusions observed might be artifacts of the procedure for separating protein and starch, semolina particles and sections of wheat endosperm cut from just below the aleurone layer were examined by electron microscopy. Both types of specimen revealed that the osmiophilic lines surrounding each piece of protein matrix had been derived originally from zones surrounding each starch granule, as in Figs. 2 and 3.

In these electron micrographs, unlike those of Seckinger and Wolf (12), the starch granules have been retained during sectioning, as evidenced by the dark staining within the granule area. Each granule is surrounded by a membrane residue located at some distance from its surface, and possibly derived from the original amyloplast membrane. Between this membrane residue and the surface of the starch granule is a band of comparatively structureless material, of varying width, which Seckinger and Wolf attributed to penetration of embedding agent into space left by shrinkage of the starch granule during fixation. However, we have observed instances in the case of Summit wheat in which the granule was only one-half the size of the area enclosed by the membrane, and, in fact, some similar areas in which there was no sign of a starch granule ever having been present. Three explanations for the presence of these areas have occurred to us:

- a) Shrinkage of the protein matrix during fixation and dehydration prior to embedding. Some freeze-etching experiments to be reported in a later communication support this view.
- b) Folding of the thin slices of starch during manipulations of cut sections prior to electron microscopy.
- c) Sectioning just above or tangentially to a starch granule.

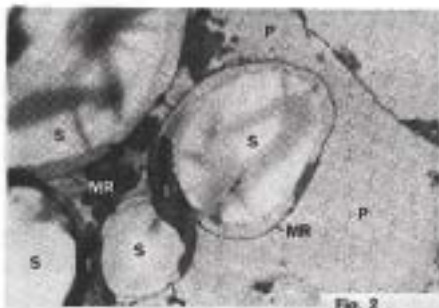


Fig. 2

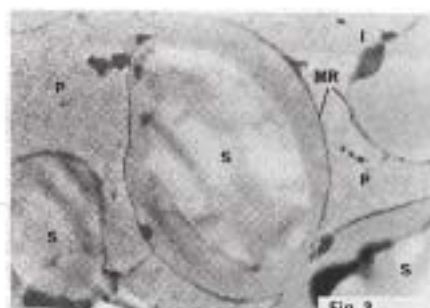


Fig. 3

Fig. 2. Transmission electron micrographs of wheat semolina particles, showing membrane residue (MR) surrounding each starch granule (S), osmiophilic inclusions (I), and storage protein (P), cv. Timgalen (18,000X).

Fig. 3. As above, cv. Summit (18,000X).

The variability in size and the frequency of distribution of these areas suggest that shrinkage of the protein matrix is the most likely explanation of the phenomenon. A curious feature was that the spaces were never present in stained sections under the light microscope, where the protein stain was always observed to completely fill the spaces between the starch granules. The presence of dark areas of osmiophilic staining within the area bounded by the membrane residue is also characteristic of many of these clear zones. The soft wheats differed from Timgalen in that contraction of the protein away from the starch granules was considerably more pronounced, suggesting both that the protein matrix in these wheats is structurally weaker, and that its adhesion to the starch granules is less. This appears to be a varietal characteristic and is consistent with the milling behavior of these wheats. While in most instances the osmiophilic line surrounding each starch granule tended to pull away from the surface and remain with the storage protein during processing, several instances were observed in which remnants of the membrane could be seen still attached to the granule surface as illustrated in Fig. 6, thus providing unequivocal evidence for its derivation.

#### Osmiophilic Inclusions

Outside the membrane structure the matrix of storage protein tends to have a granular appearance with osmiophilic inclusions of two principal types. Some of these are dark featureless areas which appear to represent bound lipid corresponding to the osmiophilic areas noted by Seckinger and Wolf (12). In addition, however, several dark areas have been noted which show ultrastructure, and which appear to be derived from cell organelles which have survived to varying degrees the process of grain maturation. Some of these inclusions are illustrated in Figs. 4 and 5.

In all samples of Timgalen wheat examined, large areas of relatively intact endoplasmic reticulum were evident (Fig. 4). These were characterized by the abundant presence of groups of ribosomes lining the chains of vesicles and canaliculi of which the endoplasmic reticulum was composed. Many structures were noted in which a spherical zone of endoplasmic reticulum had been sectioned

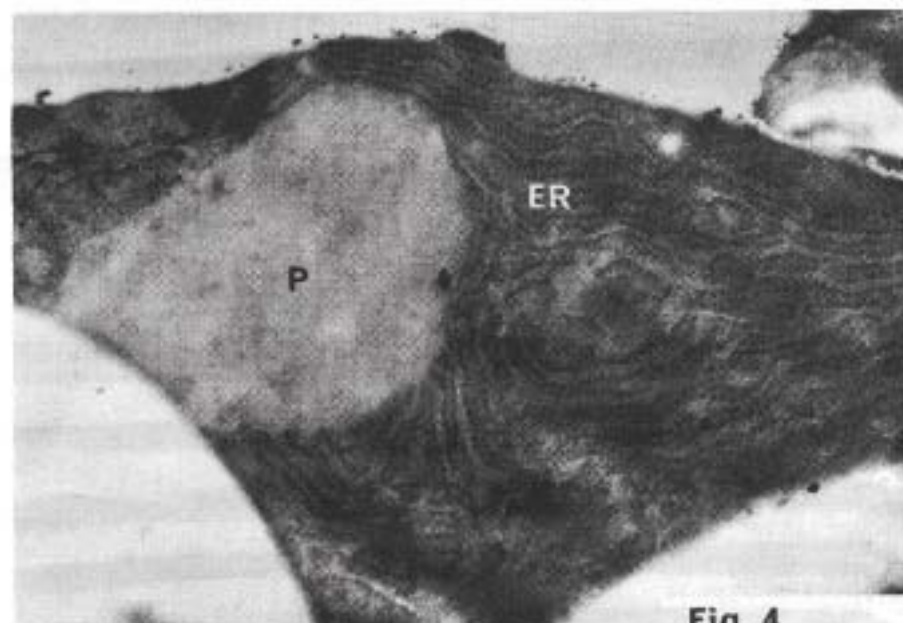
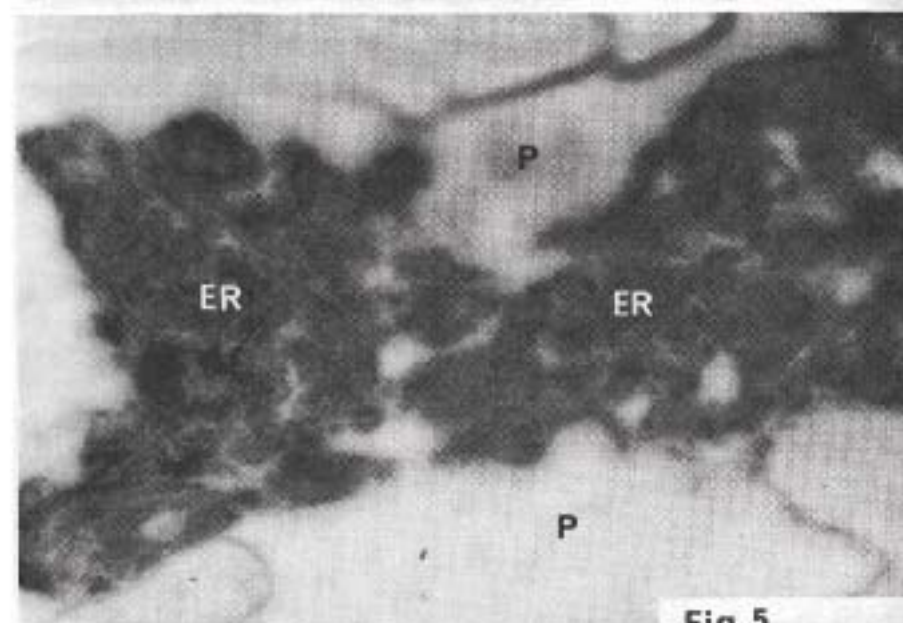
**Fig. 4****Fig. 5**

Fig. 4. Timgalen wheat, showing relatively intact areas of endoplasmic reticulum residues (ER) (60,000X).

Fig. 5. Heron wheat, showing diffuse and less ordered areas of endoplasmic reticulum residues (ER) (60,000X).

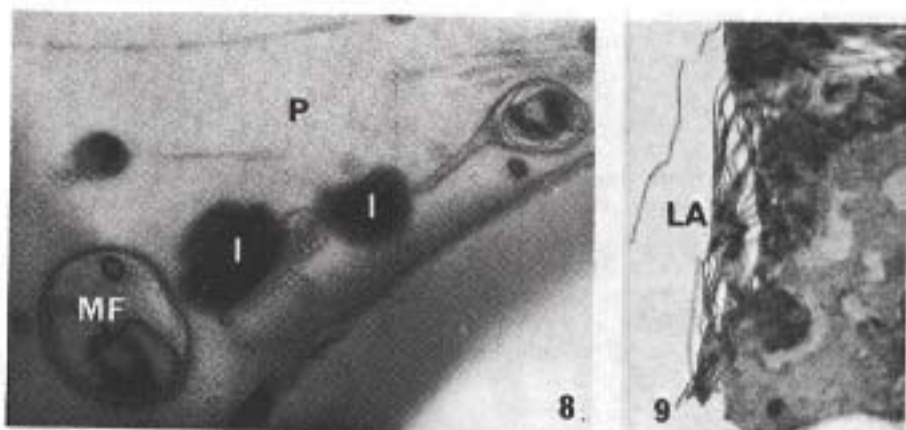
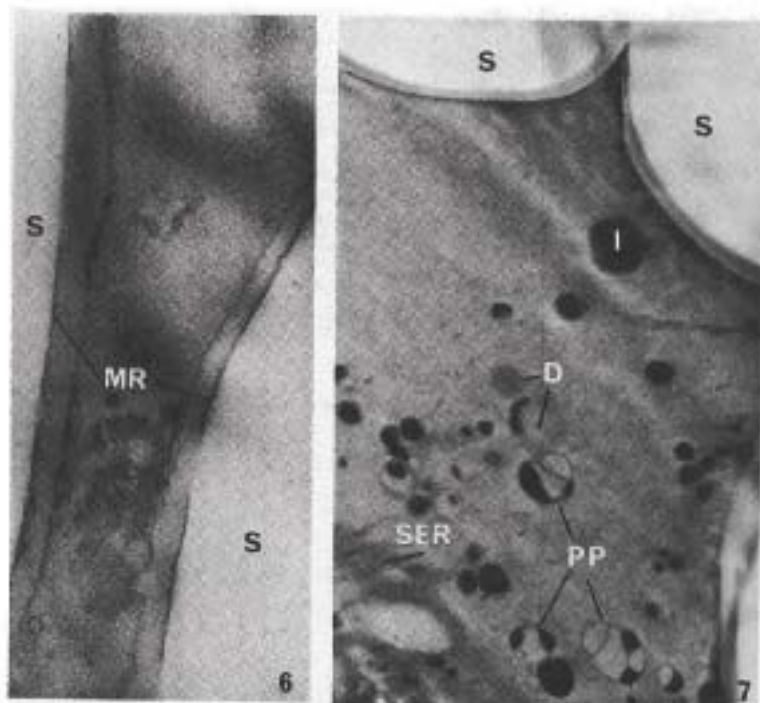


Fig. 6. Detail of starch-storage protein interface showing remnants of amyloplast membranes (MR) attached to starch granules (S) (50,000X).

Fig. 7. Transmission electron micrograph of storage protein (cv. Falcon) showing residues of cell organelles. D = dictyosome-like structure; PP = plastids; SER = smooth endoplasmic reticulum; I = cellular inclusion of unknown derivation (24,000X).

Fig. 8. Myelin figures (MF) frequently encountered in transmission electron micrographs of storage protein (P), cv. Heron; other inclusions (I) and sections of smooth endoplasmic reticulum (top right area of photo) are also illustrated (60,000X).

Fig. 9. Laminar artifact (LA) occasionally observed in storage protein, cv. Heron (23,000X).



to show a series of concentric channels leading into a central area of storage protein. A structure of this type was noted by Jennings et al. (4), but in our specimens there appeared to be larger numbers of channels and there was no evidence of a membrane surrounding the central protein body. The appearance suggests that deposition of storage protein in the developing grain is by excretion into a central area. Further work on material at the later stages of development will be necessary to determine the transition between protein-body formation (2,4) and the type of storage-protein deposition noted here.

In all other wheat varieties examined, the residual structure of the endoplasmic reticulum was much less marked, and the quantities present were much smaller. Under conditions of similar nitrogen status, Timgalen wheat is capable of reaching higher protein nitrogen contents than the other wheat varieties examined. It is possible that this ability is related to the extent to which the endoplasmic reticulum is able to retain its conformation and remain functional during the maturation process.

Other structures which have been encountered are illustrated in Figs. 7-9. Cytoplasmic inclusions of the types marked D and PP in Fig. 7 were frequently observed. While these have no counterpart in the electron micrographs of Buttrose (2) and of Jennings et al. (4), they do bear a close resemblance to the proplastids and dictyosomes (Golgi apparatus) discussed by Porter (18), and may therefore have been derived from these organelles in the developing grain. Myelin figures were frequently observed, and are illustrated in Fig. 8. One artifact occasionally encountered was a tendency for the storage protein in some sections to assume a regular stranded appearance. An extreme form of this is shown in Fig. 9. Several inhomogeneities (I, Figs. 2,5,8,9) of the type noted by Buttrose (2) were also observed.

Residues of mitochondria have not been observed in mature endosperm cells although they were frequently noted in the cells of the aleurone layer.

### CONCLUSIONS

Determination of the nature of these osmiophilic inclusions is of considerable importance to an understanding of the structure and behavior of gluten in wheat-flour doughs. Gluten itself is a lipoprotein complex (19,20) capable of forming a membranous structure surrounding gas bubbles and starch granules during dough mixing and panary fermentation. The unequivocal demonstration of endoplasmic reticulum and other membranous residues in mature endosperm cells therefore explains the origin of lipid, both free and bound, and possibly also of part of the glutenin fraction in wheat flour and dough. Other lipid is undoubtedly contributed by transfer from the embryo during the process of roller milling. Such lipid, however, would be expected to have a diffuse and structureless appearance, and to be readily extracted by the nonpolar solvents usually used to remove "free" lipids. "Bound" lipids, on the other hand, will be those closely associated with cell organelles which still maintain their membranous structure reasonably intact. Clearly recognizable mitochondria have been seen in aleurone cells separated and examined by the same process of solvent separation, fixation, and embedding, showing that the procedures used here do not disrupt structures composed of lipoprotein membranes.

However, the possibility that the various treatments to which the protein has been subjected may form artifacts having a bilayer structure must be seriously considered. The cytoplasmic inclusions shown in Figs. 8 and 9 suggest that rearrangement and interaction of lipid and protein during the processes of fixation and staining can occur within the storage-protein matrix. The relative ease with which such structures are formed under these conditions is relevant to the process of gluten formation from wheat storage protein. These and other observations also suggest that the glutenin fraction of gluten is derived from at least three sources:

- a) From a high-molecular-weight fraction present in the storage protein of the developing and mature grain (21);
- b) By intermolecular association of protein components with lipid when water is added to flour during dough preparation; and
- c) By degradation of membranes and cell organelles in the developing and maturing wheat endosperm.

Attempts to quantitatively assess the relative contributions from each of these groups to the total glutenin content are likely to be difficult, but are essential to a complete understanding of the role of protein in determining wheat quality.

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