The Nature of the Starch-Protein Interface in Wheat Endosperm

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ABSTRACT

Wheat varieties differ in the vitreosity and hardness of their mature air-dry endosperms. Micropenetrometer hardness testing indicated little difference between either starch or protein from different varieties. The conclusion is that the nature of the starch and storage-protein interface differs between hard and soft varieties. Information on this interface has been obtained by light microscopy coupled with soluble protein extraction and protein staining, by transmission electron microscopy, by scanning electron microscopy, by freeze etching, and by fluorescent antibody staining. The entire area between starch granules is filled with material staining as protein. Water-soluble proteins are confined to a position immediately surrounding starch granules, and this area is capable of rapid swelling on hydration. There is evidence that residues of the original amyloplast membranes, as well as those of endoplasmic reticulum, exist around starch granules. The soluble proteins associated with starch granules form an electrophoretically complex group. Associated with them are carbohydrates giving rise to glucose on hydrolysis. The total water-soluble material appears to play the role of a cementing substance between starch granules and storage protein. It is likely that through the amount and composition of this material the genetic control of grain hardness is expressed.

The major constituents of wheat endosperm cells are starch granules and storage protein. Starch granules develop within plastids (1), and the storage protein has also been reported to be deposited within a membrane-enclosed compartment (1,2). Thus, during grain development, the starch granules are separated from storage protein by lipoprotein membranes. It is not known, however, whether these membranes persist in some form in the completely mature cell. In thin sections of

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such cells, structures resembling membrane remnants have been reported to occur at the starch-protein interface, although they have not been unequivocally identified (3).

Wheat varieties differ in the vitreosity, hardness, and milling behavior of their endosperms. There are two possible explanations: either the physical hardness of the storage components (starch and protein) differs among varieties, or adhesion between protein and starch increases with increasing hardness of wheats. Experiments have now been done to decide this question, and to characterize the starch-protein interface which plays an important part in determining grain hardness (4,5).

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L.) of the Australian varieties Timgalen, Falcon, Heron, and Olympic was obtained from variety trials conducted by the Agricultural Research Institute, Wagga Wagga, N.S.W., and the North West Wheat Research Institue, Narrabri, N.S.W. Hard and soft variants of the varieties Heron and Falcon (4,5) were also generously provided by the Agricultural Research Institute. Gabo wheat was grown at the Waite Agricultural Research Institute, Adelaide, S.A. All wheats were milled to approximately 70% extraction on a Buhler experimental mill.

Preparation of Starch and Storage Protein

Starch and storage protein were separated by air classification and solvent suspension in mixtures of chloroform and benzene (6,7) as previously described (3,8). The purified products were extracted four times in stoppered centrifuge tubes with sodium pyrophosphate (0.01M, pH 7.0). The solutions obtained from each series of extractions were combined and their protein content determined by the method of Lowry et al. (9).

Hardness Testing

Purified starch and storage protein preparations were dispersed in a polyester-type resin (Astic, supplied by John Morris Pty. Ltd., 63 Victoria Ave., Chatswood, N.S.W. 2067) and polished according to the method of Zeidler and Taylor (10). The embedded and polished specimens were subjected to hardness testing using a micropenetrometer (Leitz Miniload hardness tester). Hardness in Vickers units was calculated from tables supplied with the instrument, or by means of the formula:

$$HV = \frac{1854 \times P}{d^2}$$

where HV = Vickers hardness in kg. per mm.^{2}, P = measuring force in pond, and d = length of the indentation diagonal in μm.

Light and Electron Microscopy

Transverse sections for examination by light microscopy were cut from blocks prepared by making two transverse cuts approximately 2 mm. apart near the center of the long axis of the grain. After fixation in glutaraldehyde (3% in phosphate buffer, 0.025M, pH 6.8) they were embedded in glycol methacrylate (11) or in

low-viscosity epoxy resin (12). Sections for electron microscopy were cut from blocks taken from the same area of the grain. These were post-fixed in osmium tetroxide and embedded in Spurr's low-viscosity resin as previously described (3).

Scanning electron microscopy was conducted with a JEOL (Japan Electron Optics Laboratory Co. Ltd.) JSM-U3 scanning electron microscope. Freeze-dried starch granules were coated with a gold layer 20 to 30 nm. thick to provide the required conducting surface.

Freeze Etching

For freeze-etching, small plugs (1 $mm.^3$) of tissue were removed from the center of the endosperm "cheeks" at a point midway along the long axis of the grain. Plugs were taken from air-dry endosperm, from endosperm that had been immersed for 4 hr. in 3% glutaraldehyde in 0.025M phosphate buffer at pH 6.8, and from endosperm that had been similarly immersed and then soaked for 16 hr. in 20% glycerol. The plugs were then placed on gold specimen discs, those of air-dry endosperm with a small drop of 100% glycerol, and plugs from the other treatments with a drop of the solution from which they were finally removed. Specimens were frozen by immersion in liquid Freon 22 cooled by liquid nitrogen, and freeze-etch replicas were obtained using a Balzers freeze-etch unit. Etching of the hydrated preparations was carried out at $-100°C.$ for 2 min.

Fluorescent Antibody Staining

Fluorescent antibody (FA) staining (13) was carried out using the techniques of Nairn (14). The antigenic material was prepared by the extraction of wheat flour (cv. Timgalen) with aqueous buffer (sodium pyrophosphate, 0.01M, pH 7.0). After dialysis against distilled water, the extracts were freeze-dried and injected into New Zealand White rabbits according to the schedule of Gell and Coombs (15). The

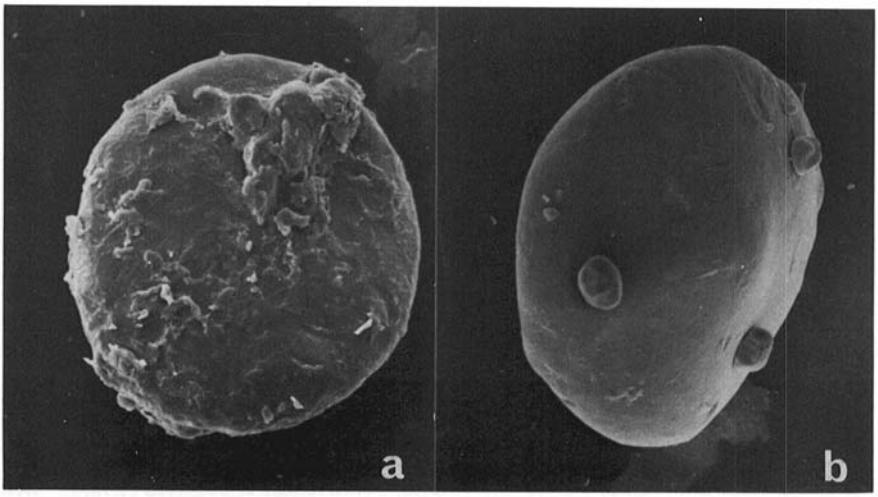

Fig. 1. Scanning electron micrographs of prime starch granules separated by sedimentation in chloroform-benzene mixtures of density 1.484 (3). a, starch granule from a hard wheat variety (Falcon); b, starch granule from a soft wheat variety (Heron).

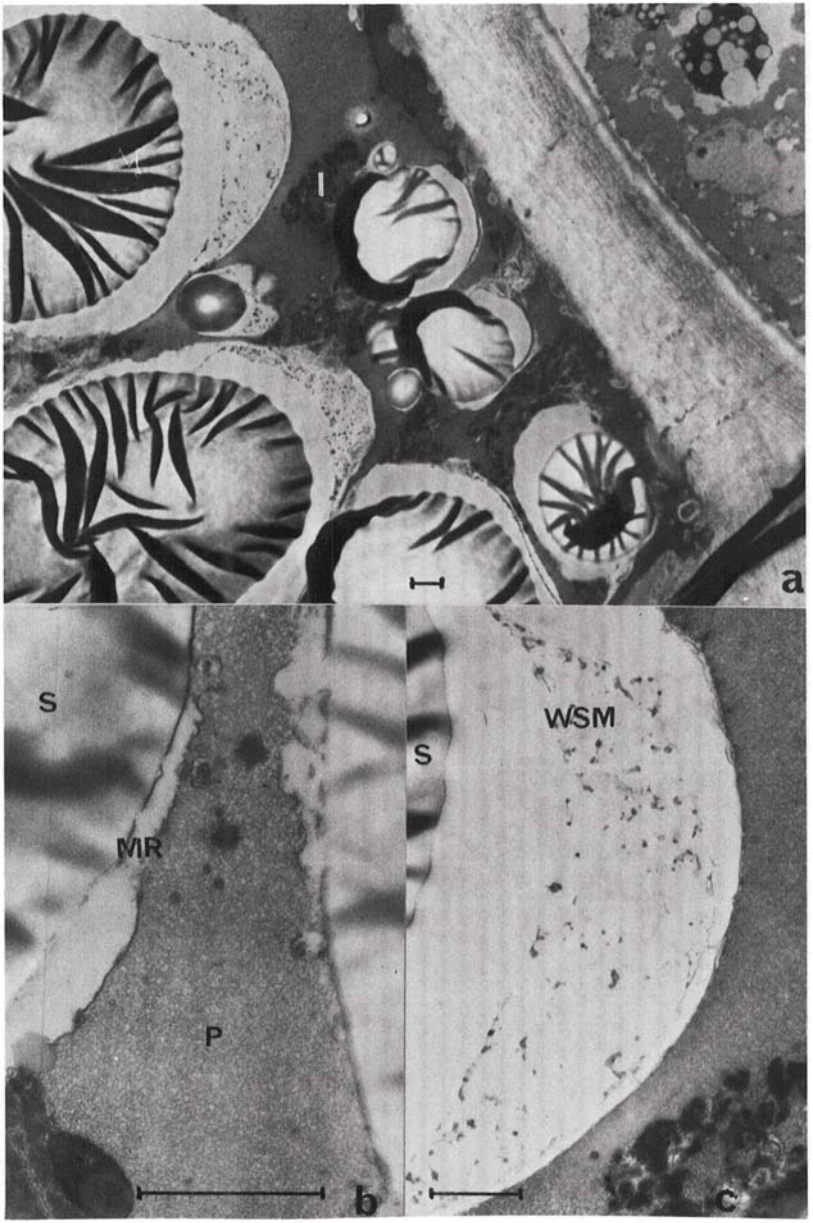

Fig. 2. Transmission electron micrograph of transverse sections of mature wheat endosperm cells. Scale lines represent 1 μm. a, Falcon (hard spring) wheat: subaleurone endosperm cells showing osmiophilic inclusions (I). b, Heron (soft spring) wheat: membrane remnant (MR) shown on either side of embedding agent-filled space. Starch granules (S) and storage protein (P) are also shown. c, Falcon wheat endosperm cell, showing possible residue of water-soluble material (WSM) between surface of starch granule (S) and storage protein (P). Membrane remnant (MR) is also visible.

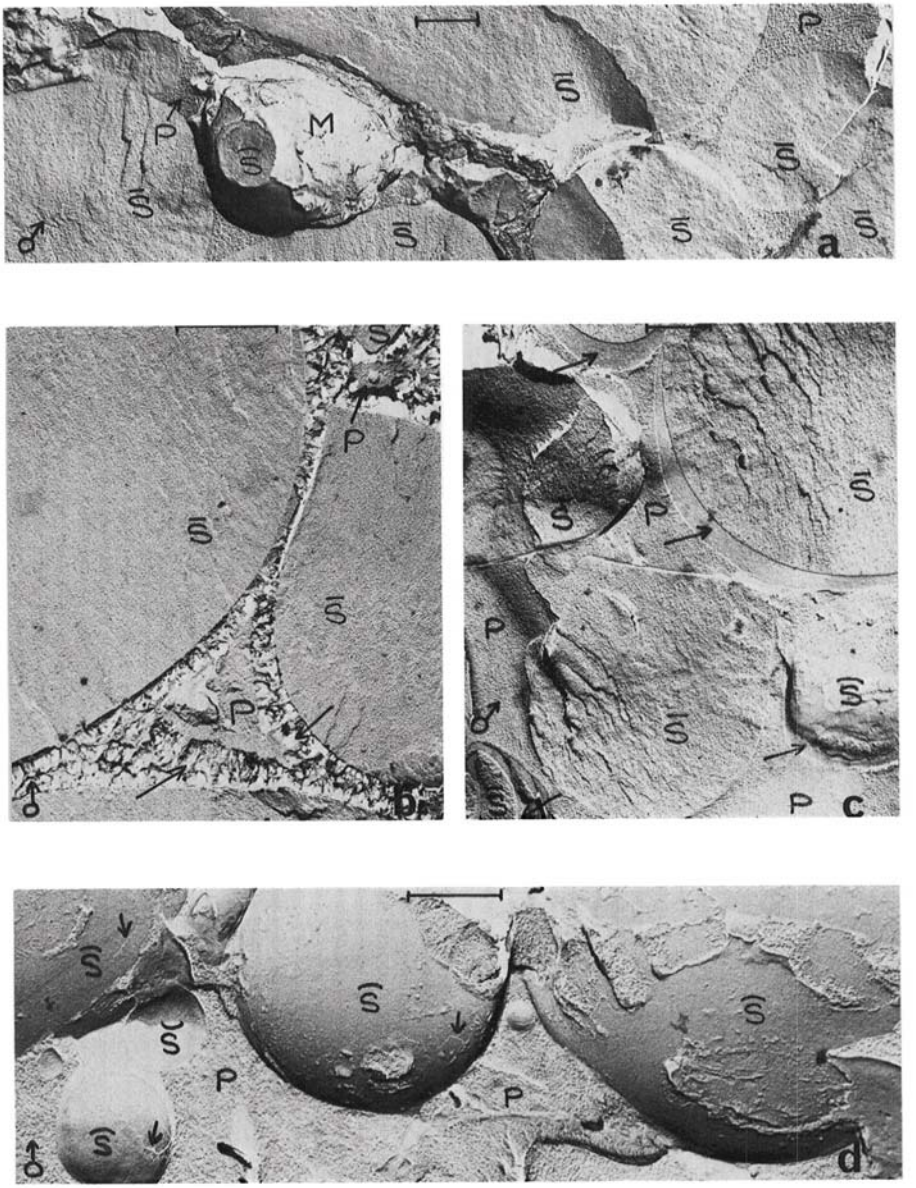

Fig. 3. Freeze-etch replicas of endosperm tissue. Scale lines represent 1 μm. S = starch granule, P = protein, M = remnants of membranes, ♂ = direction of shadowing, — = cross-fracture, ⌢ = convex surface fracture, ⌣ = concave surface fracture. a, Air-dry Gabo endosperm frozen in 100% glycerol. Membrane remnants cover half of the convex fractured granule. b, Timgalen endosperm after glutaraldehyde treatment. Arrows indicate ice crystals. c, Gabo endosperm after glutaraldehyde-glycerol treatment. Arrows indicate spaces between granules and storage protein. d, Soft Falcon endosperm after glutaraldehyde-glycerol treatment. Arrows indicate where the fracture plane has changed level.

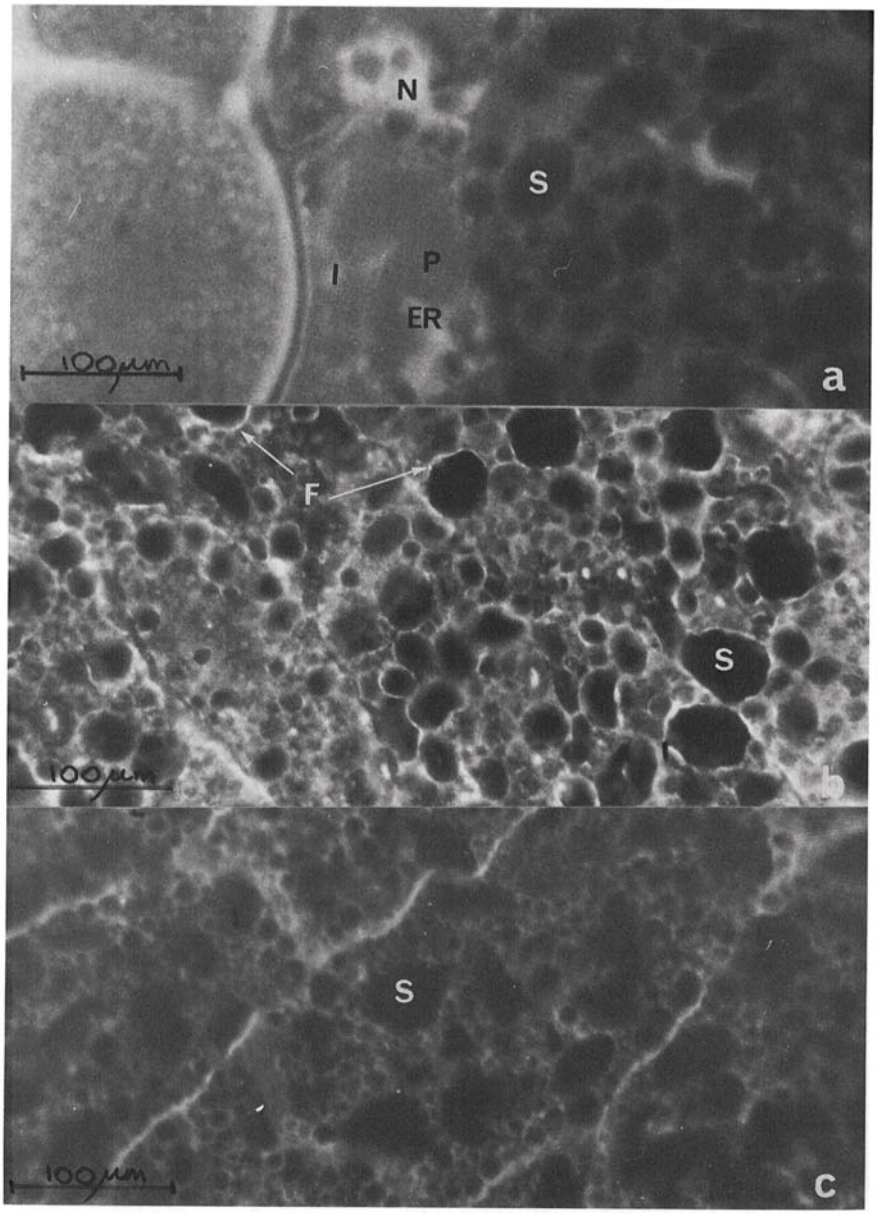

Fig. 4. Localization of water-soluble proteins in wheat endosperm (cv. Timgalen) by FA staining. P = storage protein, S = starch granules, I = nuclear remnant inclusions. a, Autofluorescence of aleurone and subaleurone endosperm cells as observed through exciter filter UG 1 and barrier filter 41. Bright field illumination. b, Immune serum labeled antibody treated section. Green fluorescence (F) is observed around each starch granule (mid-endosperm cells). Dark field illumination. c, Fluorescein-labeled nonimmune serum treated control section. Dark field illumination.

primary immunizing dose (10 mg.) was emulsified with Freund's complete adjuvant and injected intramuscularly. After 14 days, boosting doses (10 mg.) dissolved in buffered saline (phosphate buffer, 0.02M; NaCl, 0.15M; pH 7.2) were given intravenously every 2 days. The animals were bled 7 and 9 days after the tenth injection.

Microimmunoelectrophoresis (16) and double immunodiffusion (17) were used to test the resulting antisera for the presence of antibodies specific to water-soluble wheat proteins.

Both plastic-embedded (3) and cryostat sections were used for FA staining with essentially the same results. Plastic-embedded sections were counterstained with increasing concentrations of eriochrome black T until a correct balance was obtained between masking of grain autofluorescence and the labeled antibody fluorescence. Polymerized plastic was removed using sodium methoxide (10% Na in MeOH). However, this gave sections which were badly distorted, and less satisfactory for FA staining than sections cut using the cryostat microtome. Nevertheless, where undistorted areas could be observed, the 1 to 2-μm. sections which could be cut from plastic-embedded material provided superior resolution to the thicker (6 to 8 μm.) sections available from the cryostat, which were difficult to record photographically. In the procedure finally adopted, slices of wheat endosperm (cv. Timgalen) with aleurone cells attached were fixed for 12 hr. in 4% formaldehyde in phosphate buffer (0.025M, pH 6.8) and washed for 24 hr. in the same phosphate buffer before being frozen for sectioning on the cryostat. The cut sections (6 μm. thick) were allowed to stand overnight before incubation with rabbit antisera at room temperature for 60 min. After washing in phosphate-buffered saline (PBS) (pH 7.1) for 60 min., the sections were incubated with fluorescein-conjugated sheep anti-rabbit globulin for 60 min. They were then washed for 60 min. in PBS, rinsed in distilled water, air-dried, and mounted in phosphate-buffered glycerol (pH 8.6). The sections were examined under a Zeiss fluorescence microscope using primary filter UG 1 and secondary filter No. 41. Photography was carried out using Ilford HP4 or Kodak Ektachrome film.

Nonspecific staining was limited as much as possible by absorbing both antiserum and conjugate with a liver homogenate. Table I summarizes the controls used to establish the specificity of staining.

RESULTS

Hardness Testing

The hardness of starch granules, fragments of storage protein, and plastic-embedding matrix are given in Table II. Values for starch and protein were

TABLE I. CONTROLS USED FOR ESTABLISHING SPECIFICITY OF STAINING IN THE FA PROCEDURE

Stain A	Stain B	Result
Saline	Labeled antibody	No green fluorescence
Nonimmune serum	Labeled antibody	No green fluorescence
Preimmune serum	Labeled antibody	No green fluorescence
Specific serum	Labeled antibody	Green fluorescence located around each starch granule
Specific serum absorbed with antigen	Labeled antibody	Green fluorescence markedly reduced

TABLE II. MICROPENETROMETER HARDNESS MEASUREMENTS ON STORAGE PROTEIN FRAGMENTS AND STARCH GRANULES SEPARATED FROM DIFFERENT WHEAT VARIETIES

Variety	Hardness (kg./mm.^{2})[a]	
	Protein	Starch
Heron	32.60	27.04
Hard Heron	33.03	27.35
Timgalen	34.36	27.88
Soft Falcon	32.86	26.79
Falcon	32.73	28.54

[a]Resin hardness: 56.78 kg. per mm.^{2}

very similar over a range of wheat varieties differing widely in particle size index (PSI) (4). Values for the plastic-embedding matrix were quite different.

Light Microscopy

A clear space around starch granules was regularly observed where tissue was embedded in low viscosity epoxy resin, but not in similarly fixed tissue embedded in glycol methacrylate. In the latter embedment, fast green- or toluidine blue-staining material filled the entire area between starch granules.

Electron Microscopy

Scanning electron micrographs showed that starch granules prepared from hard and soft wheats by solvent sedimentation differed in the amount of material adhering to their surfaces (Fig. 1). In transmission electron micrographs, the additional presence of what appeared to be a membrane remnant surrounding each starch granule could be discerned (Fig. 2, a and b). In some cases, this was attached to the surface of the granule; in other cases, it was torn from this position and adhered to the storage protein matrix as shown in Fig. 2b.

Between the membrane remnant and the granule surface was a space of varying dimensions that appeared to contain only embedding agent. The width of this space was reasonably constant within a sample, but was quite variable between different samples and varieties. Other cereals, notably barley, rye, and rice, failed to show such spaces under similar conditions of fixation and embedding, suggesting that those observed in wheat might be artifactual in nature.

In several instances, the interfacial gap contained deposits that might have been associated with the presence of material in this region prior to fixation. The appearance of these deposits is shown in Fig. 2c.

Areas of intense osmiophilia (marked I) have also been noted in thin sections (Fig. 2a). In many cases, these showed the characteristic structure of endoplasmic reticulum, although in a highly degraded state. Such areas were frequently seen to be in close association with the membrane remnant surrounding a starch granule.

Freeze Etching

During the freeze-etching process, the freeze fracture passed either through the starch granule (Fig. 3a) or around it (Fig. 3d). Examples of both were found in any given sample, but their relative frequency depended upon variety (Table III). The proportions of cross fractures observed roughly paralleled the hardness of the wheat as judged by PSI measurements (4).

TABLE III. RELATIONSHIP BETWEEN PSI (4) AND FRACTURE TYPE IN THE FREEZE-ETCHING PROCESS

Variety	Surface Fracture %	Cross Fracture %	PSI[a]
Falcon	2	98	12.5
Hard Heron	27	73	12.9
Gabo	22	78	14.3
Timgalen	37	63	18.0
Summit	59	41	26.0
Soft Falcon	68	32	28.6
Heron	50	50	29.5

[a]The lower the PSI, the harder the wheat.

Freeze-etch replicas of tissue frozen direct with glycerol showed a homogeneous texture between starch granules at many points, and at others showed what are interpreted as remnants of lipoprotein membranes and lipid deposits (marked M in Fig. 3a). Tissue treated with glutaraldehyde before freezing gave replicas of ice crystals immediately surrounding the starch granules and separating them from islands of storage protein (Fig. 3b). This hydrated layer was seen more clearly after 20% glycerol had been used as an antifreeze agent (Fig. 3c); the space separating starch granule and protein lay below the plane of fracture indicating that water had sublimed away in that area. Figure 3c is a selected area; the majority of starch granules in similar replicas had no such surrounding spaces (see granule at center bottom of Fig. 3c).

The convex faces of starch granules resulting from freeze fracture were smooth in texture except where they carried some adhering material and where starch within the granule was exposed. Adhering material could have a layered structure (Fig. 3d). Corresponding concave fracture faces of granules were smooth, except where a portion of underlying "cytoplasm" was exposed.

Extraction of Water-Soluble Proteins

Several series of experiments were conducted in which wheat cross-sections, approximately 1 mm. thick, were exhaustively extracted with distilled water, 2M urea, or the acetic acid-urea-CTAB (AUC) solvent of Meredith and Wren (18), followed by glutaraldehyde fixation and embedding. In no case could any differences be observed by light microscopy between the extracted cross-sections and unextracted controls. The results of examination by electron microscopy were not easy to interpret, but suggested that adhesion between starch and protein was loosened by extraction with both water and 2M urea. Sectioning of extracted samples for electron microscopy was particularly difficult, due to compressional effects. Extraction, even with the AUC solvent, was incomplete, as judged by staining and microscopic examination. When the extracts themselves were examined, it was clear that some protein had been dissolved, but it was impossible to assay it quantitatively.

When separated starch granules and storage protein fragments, isolated by the solvent suspension procedure, were extracted with aqueous buffers (e.g., 0.01M sodium pyrophosphate, pH 7.0), marked differences in the proportions of

water-soluble proteins associated with each were observed (Table IV). A very high proportion of the total protein associated with starch granules was buffer-soluble, and therefore of the albumin-globulin type. For soft wheat varieties, which had starch granules almost completely free of visibly adhering storage protein (Fig. 1), the proportion of water-soluble protein in the starch fraction approached 90%, although, as shown in Table IV, the total amount of protein present in these cases is quite low. Buffer extracts from separated starch granules contained both carbohydrate and a mixture of proteins, as judged by acrylamide gel electrophoresis. The carbohydrate yielded only glucose on hydrolysis.

TABLE IV. PROPORTIONS OF WATER-SOLUBLE PROTEINS PRESENT IN STARCH AND STORAGE PROTEIN PREPARATIONS FROM VARIOUS WHEAT VARIETIES[a]

Sample	Total Protein % dry wt.	% Total Protein Extracted by Buffer[b]
Storage protein:		
Gamenya	94.6	5.84
Heron	92.6	8.38
Timgalen	90.9	4.73
Starch:		
Gamenya	0.9	87.4
Heron	1.0	70.0
Timgalen	3.0	60.2

[a]Protein extracted by 0.01M Na pyrophosphate, pH 7.0, is expressed as a percentage of the total protein present, average of three runs.
[b]0.01M Na pyrophosphate.

Fluorescent Antibody Staining

This technique provides in theory a very sensitive and specific method for the detection and localization of the water-soluble proteins *in situ*. Problems of grain autofluorescence may be overcome by counterstaining or by the use of appropriate filters. Figure 4a illustrates the autofluorescence through the filter system finally selected. The aleurone cell walls show a very bright blue autofluorescence which is particularly difficult to quench. Storage protein gives a deep blue autofluorescence, while the nuclear remnants, endoplasmic reticulum, and several other cellular inclusions (3) emit a bright yellow color under these conditions. Starch granules are nonfluorescent under ultraviolet light. The reaction between the labeled antibody and specific antigen (water-soluble protein) is shown in Fig. 4b, which illustrates that the pale green fluorescence (marked F) due to the product of this reaction occurs specifically around each starch granule. This effect can be noted in both subaleurone and midendosperm portions of the grain. The concentration of fluorescence appears to be higher in the latter area, possibly because of the higher concentration of starch granules. Figure 4c shows the effect of incubating the section with fluorescein-labeled nonimmune serum. No specific staining is seen to be associated with starch granules.

DISCUSSION

Results of hardness testing suggest that individual storage components do not differ in hardness between varieties, but that the adhesion between starch and

protein does differ. This is supported by scanning electron microscopy and freeze-etching results. In hard wheats, fractures during milling tend to pass along endosperm cell walls to yield clean, well-defined particles. Fracture through cell contents in these wheats, when it occurs, involves both starch granules and storage protein, resulting in a high proportion of damaged and broken starch granules. Because of the lower adhesion between starch and protein, soft wheats tend to release starch granules more freely during milling, with fractures occurring around rather than through granules. This results in much less starch damage in the latter case.

Both transmission electron microscopy and freeze-etching studies have demonstrated the presence of membrane remnants around starch granules. These may represent the original amyloplast membrane of the developing starch granule (1,3,19), although pockets of other membrane remnants have been observed (Fig. 3a, center). The fact that both convex and concave fracture faces in freeze-etch replicas are smooth suggests that the fractures have passed through a lipid phase. This lends additional support to the hypothesis that some form of the original amyloplast membrane occurs at this point.

Plastic-filled spaces around starch granules, observed in thin sections, appear to be artifacts in the light of the freeze-etch results. This confirms the opinion of Seckinger and Wolf (20). Their presence is probably the result of swelling and contraction phenomena associated with the fixing, dehydration, and embedding procedures (21). Since these areas are stained by protein stains when examined by light microscopy, it is possible that they contain water-soluble proteins which are cross-linked and stabilized by the glutaraldehyde fixative used. This is confirmed by the presence of a fine network of material sometimes observed in the interfacial area (Fig. 2c).

Previous workers (22,23) have drawn attention to the enrichment in water-soluble proteins which occurs in starch-rich fractions obtained by air classification or solvent sedimentation, and our results show that a complex mixture of these proteins may be detected in close proximity to starch granules. Some of these may be residues of the enzymes and associated factors involved in starch synthesis in the developing grain.

The production of only glucose on hydrolysis of the carbohydrate material present in this area suggests that it consists mainly of incompletely synthesized starch chains of various degrees of complexity. Further work will be necessary to identify and adequately characterize these substances. Of particular importance would be the recognition of a substance or group of substances present in higher concentration in hard wheats whose production, like that of grain hardness (5), is genetically controlled.

The following conclusions are made.

1. Carbohydrate chains at the surface of the starch granule are in direct contact with components of the dehydrated stroma which was originally enclosed within the developing amyloplast (1). Several proteins, enzymes, small sugars, incomplete amylose and amylopectin chains, amino acids, and other components may be expected to occur in this zone.

2. Surrounding this zone, and generally enclosing each starch granule, is the lipid-rich remnant of the amyloplast membrane. This may be regarded as the primary zone of separation between starch and storage protein.

3. Outside this residue the storage protein may come into direct contact with the

surface of the membrane remnant. Frequently areas of endoplasmic reticulum remnants occur at this point.

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