

## ULTRASTRUCTURAL CHANGES IN WHEAT-FLOUR PROTEINS DURING FIXATION AND EMBEDDING

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### ABSTRACT

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A soft wheat flour was examined by scanning electron microscopy during preparation—osmium fixation, ethanol dehydration, and methacrylate embedding—for transmission electron microscopy. Proteins were modified to form a lamellar and fibrillar matrix. The amount of protein lost by solubilization in fixative, ethanol, and methacrylate solutions was small.

Transmission electron microscopy (TEM) has been applied in recent years to the study of cereal proteins and lipoproteins. Thus, in mature wheat endosperm and flour, some structures have been characterized (1-5)—lipid globules, smooth and compact fragments essentially protein in nature, and fibrillar lipoprotein components. A better understanding of artifacts caused by the sample preparation process is needed in order to improve the interpretation of electron micrographs. Scanning electron microscopy (SEM) requires less sample preparation and, therefore, is useful in studying this problem (6). SEM showed that free flour lipids are aggregated into globules during aqueous osmium fixation (7). This paper reports an SEM study on the structural changes in flour proteins produced by the fixation, dehydration, and embedding processes. The amount of protein lost during the various preparative treatments was determined.

### MATERIALS AND METHODS

#### Fixation, Dehydration, and Embedding Processes

The different stages of sample preparation are summarized in Table I. Industrial flour obtained from soft wheat varieties was used. Flour was fixed with 1% OsO<sub>4</sub> in 0.1M phosphate buffer at pH 7.2, for 30 min to 1 hr at 20° C (A), and then carefully washed in the same buffer. A blank (B) was obtained by putting flour into buffer alone under the same conditions as described above. Samples were dehydrated by passage through 50, 70, and 100% ethanol at 20° C,

15 min each, followed by 12 hr in 100% ethanol at 4° C (C, C<sub>1</sub>). In addition, initial flour was directly treated with 100% ethanol for 12 hr at 4° C (C<sub>2</sub>). Embedding medium was a methyl-butyl methacrylate mixture. After passage through a solution containing equal parts of 100% ethanol and embedding medium (30 min), the samples were impregnated with methacrylate (two changes in 1 hr) and methacrylate containing 1% benzoyl peroxide (12 hr at 4° C) (D). Polymerization was attained in 15 to 24 hr at 50° C. Both ultrathin sections and sections several microns thick (E) were made on an ultramicrotome. Ultrathin sections were examined with a Siemens Elmiskop I transmission electron microscope.

#### SEM

Samples were taken at the different stages of preparation (A, B, C, C<sub>1</sub>, C<sub>2</sub>, D, and E). After dehydration under vacuum, all the samples were coated with a 200 Å layer of carbon and gold before examination with a JEOL JSM 50 A scanning electron microscope.

#### Evaluation of N Lost during Preparative Treatments

The assays were made on the basis of 500 mg of flour and 7 ml of reactive. After centrifugation, the N contents of the supernatant solutions were determined by the Kjeldahl micromethod. The solutions containing 100% ethanol or methacrylate were evaporated before adding the concentrated sulfuric acid. Distillation was in an excess of 4% boric acid and titration was carried out with 0.02N HCl, in the presence of Tashiro reagent.

### RESULTS

#### SEM

Differences in the lipoproteins were evident between the OsO<sub>4</sub> fixed flour (Fig. 1A) and the unfixed flour (Fig. 1C). In the fixed flour, lipid globules described previously (7) were visible at the starch granule surface. The protein matrix was less compact and lamellar in structure. Flour treated with buffer only (Fig. 1B) appeared more similar to the original flour. When the flour was fixed and then progressively dehydrated by ethanol (Fig. 2A), there was an enhancement of the

TABLE I  
Treatment Conditions for Various Samples

Sample	Treatment					Sections
	OsO <sub>4</sub>	Buffer	Ethanol 50, 70%	Ethanol 100%	Methacrylate	
Flour	-	-	-	-	-	-
A	+	+	-	-	-	-
B	-	+	-	-	-	-
C	+	+	+	+	-	-
C <sub>1</sub>	-	+	+	+	-	-
C <sub>2</sub>	-	-	-	+	-	-
D	+	+	+	+	+	-
E	+	+	+	+	+	+

fibrillar and lamellar structure of proteins. Lipid globules were still present.

In order to better understand the action of ethanol, flour treated directly with 100% ethanol was compared with flour hydrated and post-dehydrated with 50, 70, and 100% ethanol solutions. In the first case (Fig. 2B), lipoproteins were unchanged compared to initial flour. In the second case (Fig. 2C), they were greatly altered, fibrillar and lamellar in structure, and in some cases stretched. The numerous globules visible at the starch and protein surfaces were sometimes

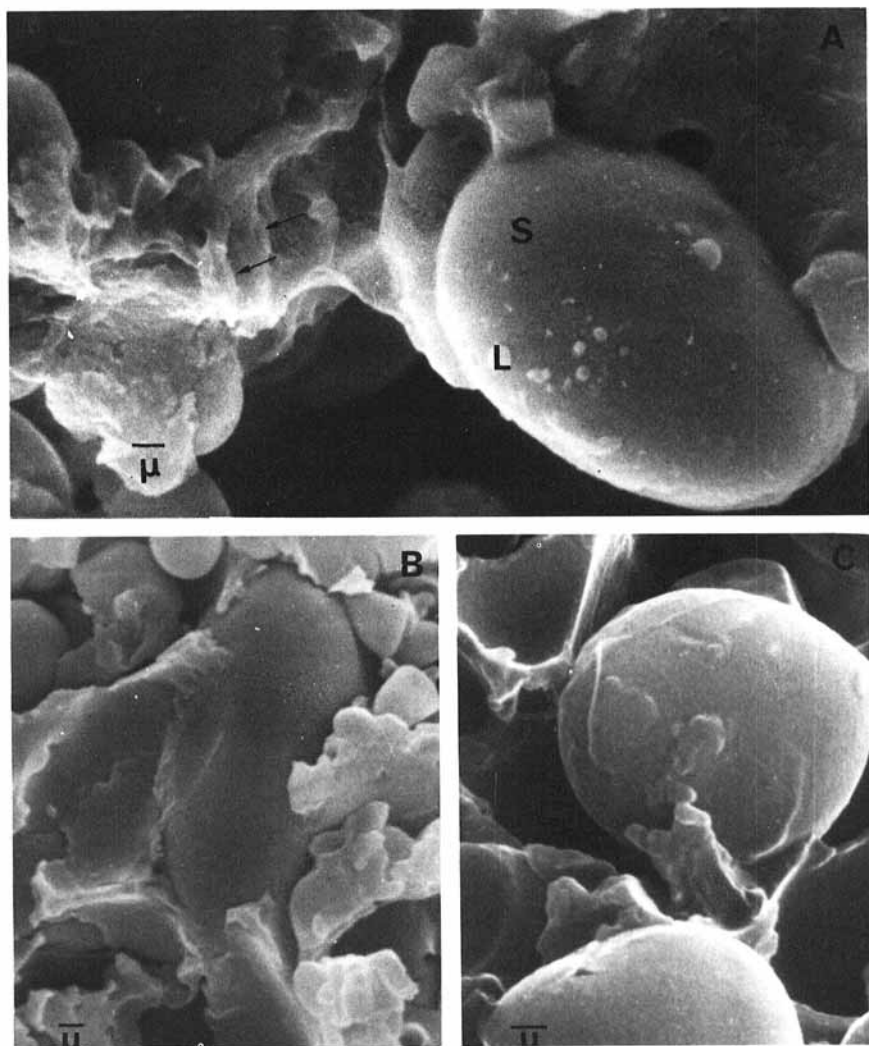


Fig. 1. Scanning electron micrographs of A: flour, osmium-fixed, showing a lamellar protein matrix (arrows), lipid globules (L), and starch granules (S); B: flour treated with buffer pH 7.2; and C: original flour.

aggregated into small chains. Not all these globules seem to be lipid fractions; many are probably formed by protein deposits.

During the passage from ethanol to liquid methacrylate, no changes in the lipoprotein structure were observed. After polymerization and sectioning, lipoprotein components appeared relatively well embedded, while starch

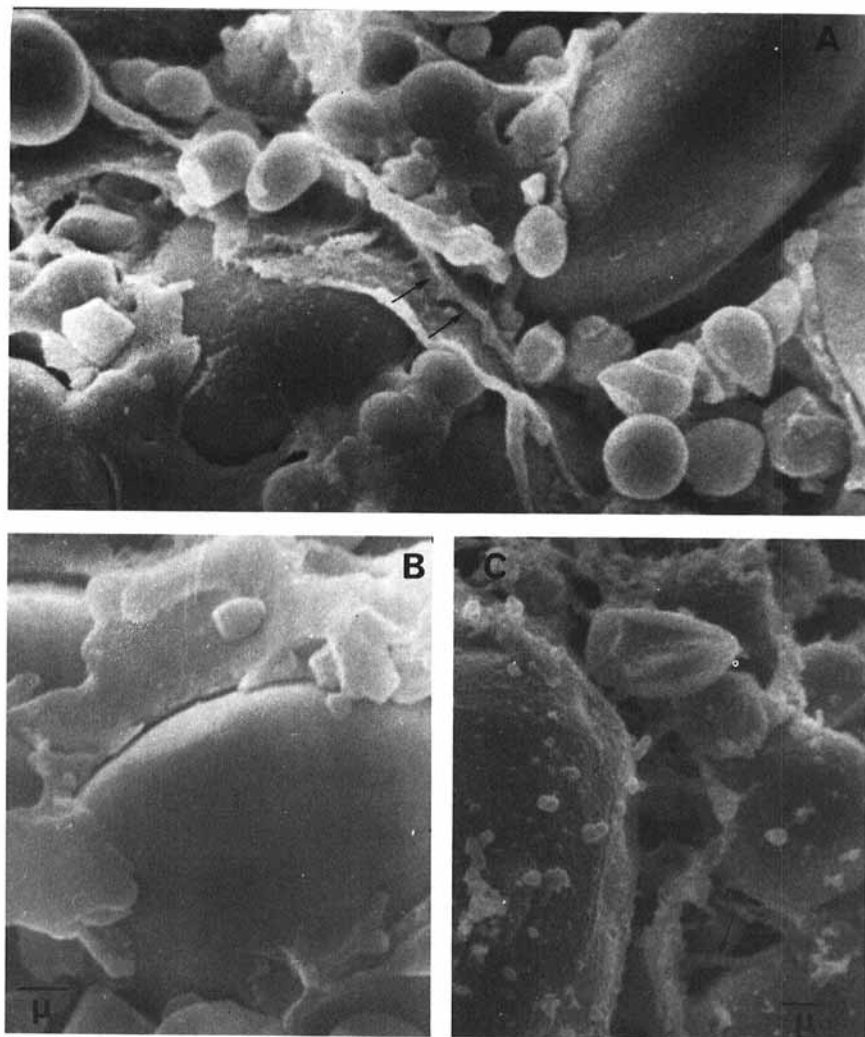


Fig. 2. Scanning electron micrographs of A: flour, osmium-fixed, then dehydrated with 50, 70, and 100% ethanol, showing a protein matrix, lamellar and fibrillar in structure (arrows); B: flour treated with 100% ethanol only; C: flour hydrated in buffer pH 7.2, then dehydrated with 50, 70, and 100% ethanol; the proteins form a fibrillar network stretched in some cases (arrows); numerous globules are present at the starch-granule surface.

granules were not adhering completely to methacrylate and protein fragments (Fig. 3A).

#### Evaluation of N Lost during Sample Preparation

Values of extracted protein N ( $N \times 5.7$ ) are reported in Table II. They show that, in normal preparation for TEM, the major part of protein is lost (about 6.5%) during fixation. The amounts released during the other treatments, dehydration, and embedding were very low (1.4 to 0.8%, respectively). In

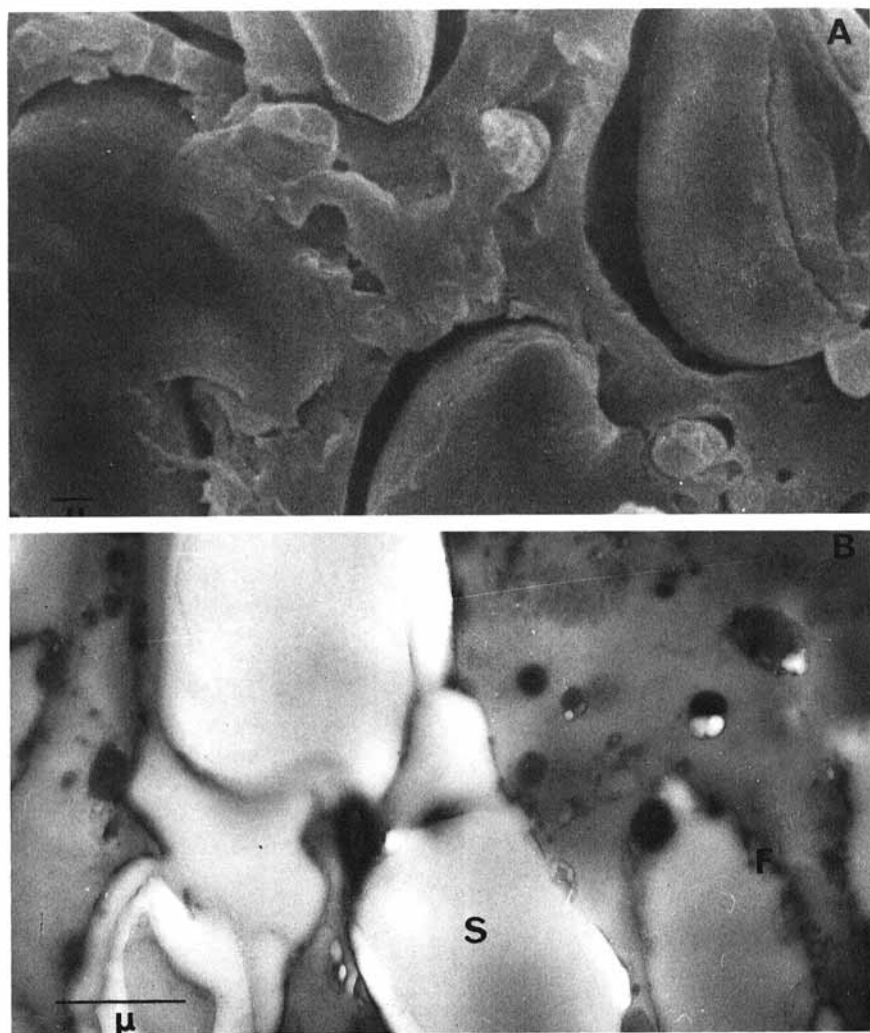


Fig. 3. Sections of fixed and embedded flour A: SEM examination, and B: TEM examination (S, starch; F, fibrillar proteins).

hydrated but not fixed flour, the protein lost in 50 and 70% ethanol was in the same order as the quantities extracted during fixation (5%), though this solvent should dissolve the gliadin fraction; this fact may be explained by the moderate conditions for ethanol dehydration. When flour was directly treated with absolute ethanol, the amount of protein N lost (0.7%) was much lower, corresponding to the values found for samples which had been submitted to the complete process of dehydration.

### DISCUSSION

These experiments evaluated total N, but not protein N specifically. Aside from this restriction, the results mentioned here give a good estimation of protein lost during the different stages of TEM sample preparation. The amounts lost were not very great—about 9% total, with 6.5% lost during fixation. The lower quantities found for buffer washing, ethanol, and methacrylate baths were insignificant. These results may be compared with those obtained by Dallam (8) on cellular fractions of animal tissues, nuclei, and mitochondria.

The microscopy and protein extraction results suggest that, during hydration in fixative, a solvation of some protein may occur. This solvation may induce a swelling and sheeting of the lipoprotein matrix. The tendency of hydrated wheat-flour proteins to form lamellar structures was reported previously (9). In this study, however, fibrillar or lamellar structures were not found in flour proteins which were hydrated only. This divergence may be explained by the fact that our samples were not cryodehydrated as those of Bernardin and Kasarda (9) after the hydration process, but were desiccated under vacuum. During desiccation, it is possible that the protein matrix returned to its approximate initial structure. However, when flour is chemically fixed, the fibrillar structure of proteins remains after desiccation. During dehydration, a solvation of some protein probably occurs in 50 and 70% ethanol. These proteins would be coagulated and layed into films as the ethanol concentration increases. This would explain the reinforcement after dehydration of the lamellar and fibrillar protein structure. When samples are not previously fixed, the fibrillar structure is still more intense. In these cases, the deposit also becomes globular-shaped. It is interesting to notice that, during treatment with only 100% ethanol, fibrillar structures did not appear.

In gluten, we observed that osmium fixation, whether or not it was followed by

TABLE II  
Protein Lost during Preparation of Flour for TEM

Sample	Per Cent Lost ( $N \times 5.7$ ) during Treatments						Total
	OsO <sub>4</sub>	Buffer	Ethanol 50, 70%	Ethanol 100%	Ethanol 100% methacrylate	Methacrylate	
Original flour	6.4	0.5	0.8	0.6	None	0.8	9.1
Hydrated flour			5				
Original flour				0.7			

ethanol dehydration, produced a complete insolubilization of proteins in 0.1*N* acetic acid (98% against about 20% for native gluten) (5). This seems to indicate that extracted fractions are probably soluble proteins rather than gluten proteins.

Microscopic observations reported here are different from those of Aranyi and Hawrylewicz (6), who did not notice structural changes in the protein matrix of fixed and dehydrated flour. These authors used a slightly different fixation procedure—glutaraldehyde + osmium tetroxide.

If SEM observations in this study are compared with those more generally obtained by TEM, it is probable that a part of fibrillar protein structures visible by TEM after osmium fixation (Fig. 3B) is formed during sample preparation. Therefore, it appears that, in addition to organized lamellar structures such as endoplasmic reticulum or lipoprotein membranes pre-existing in endosperm cells, interaction products of various protein or lipoprotein components are present in the fibrillar networks. This interpretation, which is in agreement with an hypothesis of Simmonds (3) concerning dough, shows the very complex nature of flour and gluten lipoprotein fractions visible under an electron microscope.

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