Wheat-Grain Morphology and Its Relationship to Dough Structure

D. H. SIMMONDS, C.S.I.R.O. Wheat Research Unit, North Ryde, New South Wales 2113, Australia

ABSTRACT

Mature wheat endosperm cells contain remnants of endoplasmic reticulum, amyloplast membranes, and other cell organelles which contribute extensively to flour lipids. During dough-mixing and gluten formation, these remnants form intensely osmiophilic inclusions which may be observed under the electron microscope. Such organelle residues are insoluble in urea solutions. On addition of water they also give rise to free lipid which interacts with protein to form an additional urea-insoluble lipoprotein fraction. Under the electron microscope, this fraction appears as osmiophilic vesicles of various types and sizes. It is postulated that these vesicles are derived from an extensive lipoprotein network originally present in fresh gluten and dough.

It has been shown previously (1,2) that mature wheat endosperm cells contain remnants of endoplasmic reticulum, amyloplast membranes, and other cell organelles which may be recognized under the electron microscope. These remnants have been implicated as one source of the relatively insoluble glutenin components of high molecular weight in wheat flour and gluten (2,3). It is also likely that they contribute extensively to the lipid content of flour, since no lipid droplets, vacuoles, or other lipid storage deposits have yet been recognized in developing or mature endosperm cells (1,4). Additional lipid deposits occur in the spherosomes of aleurone tissue (5) and in the epithelial and parenchymal cells of the scutellum (6). Admixture of these tissues with endosperm cells during milling could thus contribute significantly to the lipid content of white flour.

The binding of flour lipids during dough-mixing has been recognized by many workers (7–13), but the sources of the lipids involved and the nature of their interaction products with flour proteins are still imperfectly understood. Evidence for the formation of these products has relied heavily on the differential extractability of the lipids with polar and nonpolar solvents. However, several workers (14–17) have described fractions extracted from gluten which contained both lipid and protein.

Several workers have examined the distribution of lipid between the gliadin and glutenin fractions of gluten, and have arrived at conflicting conclusions. Generally the glutenin fraction has been claimed to contain the greater concentration of lipid (8,18,19). However, Ponte et al. (20) found that when gliadin and glutenin were prepared by the classic procedure involving 70% ethanol, the major proportion of the lipid was associated with the gliadin fraction. In their discussion they raise the possibility that the ethanol treatment may have caused a redistribution of the flour lipids.

Membraneous structures in general, and endoplasmic reticulum in particular, have been shown to contain high proportions of lipids (21). It is possible that these sources provide the bulk of the polar lipids responsible for the production, during dough-mixing, of the insoluble lipoprotein described in the preceding paper (22). In this communication, the properties of this insoluble fraction have been further

Copyright © 1972 American Association of Cereal Chemists, Inc., 3340 Pilot Knob Road, St. Paul, Minnesota 55121. All rights reserved.

examined and attempts have been made to establish its relationship with the ultrastructure of flour-water doughs.

MATERIALS AND METHODS

Gluten and purified storage protein (2) were prepared from pin-milled flour of Timgalen (hard) and Heron (soft) wheats, as described in the preceding paper (22).

Preparation of Samples for Electron Microscopy and Lipid Analysis

Reconstituted Samples. Portions of storage protein (500-mg.) were treated with distilled water or lipid, according to the procedures previously described (22).

Density-Gradient Centrifugation. Samples (400-mg.) of storage protein, gluten, and of storage protein subjected to the various water and lipid treatments previously described, were dispersed into 4M urea containing 20% sucrose (10 ml.), and 2-ml. portions were layered above a stepwise gradient 20 to 60% in sucrose. After centrifugation (110,000 X g, 60 min.), the insoluble fraction had formed a thin band at the interface between 50 to 60% sucrose. In some experiments an additional band formed at the interface between 40 to 50% sucrose. The remainder of the gradient was optically clear and was removed by peristaltic pumping. The banded material was suspended in distilled water, recovered by centrifugation (2,000 X g, 20 min.), and fixed in glutaraldehyde (3% in 0.025M phosphate buffer, pH 6.8) followed by osmium tetroxide (2% in the same buffer) for electron microscopy (3).

Preparation of Dough. Straight-run flours milled experimentally from the wheat varieties Timgalen, Falcon, Heron, and Summit, and a commercially milled New South Wales flour of well-balanced mixing characteristics (50 g.) were mixed with sodium chloride (1 g.) and water in a Brabender Farinograph to optimum consistency (500 to 600 B.U.). Dough samples were taken at this point. In experiments with defatted flour, lipid was removed from the commercial flour by exhaustive extraction with either petroleum ether (boiling range 40° to 60°C.) or chloroform. Solvent was removed by air-drying.

Hydrolysate Lipid Determinations

The standard AOAC procedure (23) was used with the following modifications: The sample to be analyzed was weighed directly into the Mojonnier tube to avoid manipulative losses, and hydrolysis of the lipid with HCl prior to extraction was continued for 1 hr. instead of 30 to 40 min.

RESULTS AND DISCUSSION

Mature endosperm cells contain numerous osmiophilic inclusions derived from cell organelles present in the developing grain (2,3).

Those most frequently encountered are areas of rough endoplasmic reticulum showing various degrees of residual organization (2), together with other membraneous residues derived from smooth endoplasmic reticulum, Golgi bodies, and various types of plastids. Other inclusions are of indefinite structure and less certain derivation. Some of the larger areas of this type have sufficient residual ultrastructure to suggest that they may have been derived by degradation of rough endoplasmic reticulum during maturation of the grain.

The effect of milling on these structures is negligible, since they are one to two orders of magnitude smaller than the flour particles resulting from this operation. However, mixing of flour with water to form a dough does cause marked changes to take place. Figure 1, a and b, shows the structures of doughs prepared from strong flours (cv. Timgalen and Falcon); section c of Fig. 1 shows the structure of a dough prepared from a weak flour (cv. Summit).

Examination of a large number of transmission electron micrographs showed that shrinkage of the protein phase away from the starch granules in the doughs prepared from strong flours is small compared with that in weak-flour doughs. Numerous osmiophilic inclusions are observed in the protein phase, but basically these are of two types. The majority (Type I) are of irregular outline, intensely staining, and vary in size from less than 1 to approximately 3 μm. across. They appear to have been derived from the endoplasmic reticulum of the mature cell, stripped of its ribosomes and most of its recognizable structure, as shown at higher magnification in Fig. 2, a. Interspersed among the Type I inclusions, but much fewer in number, are some osmiophilic objects, circular in section and approximately 1 to 3 μm. in diameter. These are illustrated particularly clearly in Fig. 2, b, and are termed Type II inclusions.

Extraction of flour with petroleum ether or chloroform prior to dough formation does not significantly alter the appearance of the Type I inclusions. However, the Type II inclusions are completely removed, and the background osmiophilia of the resulting dough is less intense. These observations are illustrated in Fig. 2, c.

On the basis of their osmiophilia, and their absence in doughs prepared from petroleum ether- and chloroform-extracted flour, it is suggested that the Type II inclusions are lipid-rich droplets. Similar inclusions have been noted in electron micrographs of chloroplast tissues (24), and in cross-sections of mature wheat grains. In doughs derived from Timgalen and Falcon flours, they appeared to carry a granular network within their structure, as shown in Fig. 3, a. However, this ultrastructure was not evident in Type II inclusions from Heron and Summit doughs (Fig. 3, b). In doughs prepared from the New South Wales commercial flour, they carried an outer layer of fine strands which could represent the products of interaction between the lipid droplet and its protein environment (Fig. 3, c).

To explain the appearance of these two types of inclusions in doughs, it seems necessary to postulate that during dough-mixing, endoplasmic reticulum and other lipid-rich membraneous remnants break down into two phases:

a) A lipoprotein phase which gives rise to Type I inclusions, and which owes its osmiophilia to the combined presence of lipid and protein; and

b) A lipid-rich phase which forms droplets (Type II inclusions) during mixing, and which gives rise to free lipid available for subsequent interaction with other protein components in the flour. Where endoplasmic reticulum structure in the mature flour is well defined (as in Timgalen), it is possible that a small quantity of this structure may remain within the lipid droplet and appear as shown in Fig. 3, a. The absence of such internal structure in Type II inclusions from Heron and Summit flours is consistent with the greater degradation, during ripening, of endoplasmic reticulum in mature endosperm cells of these wheat varieties (2). The

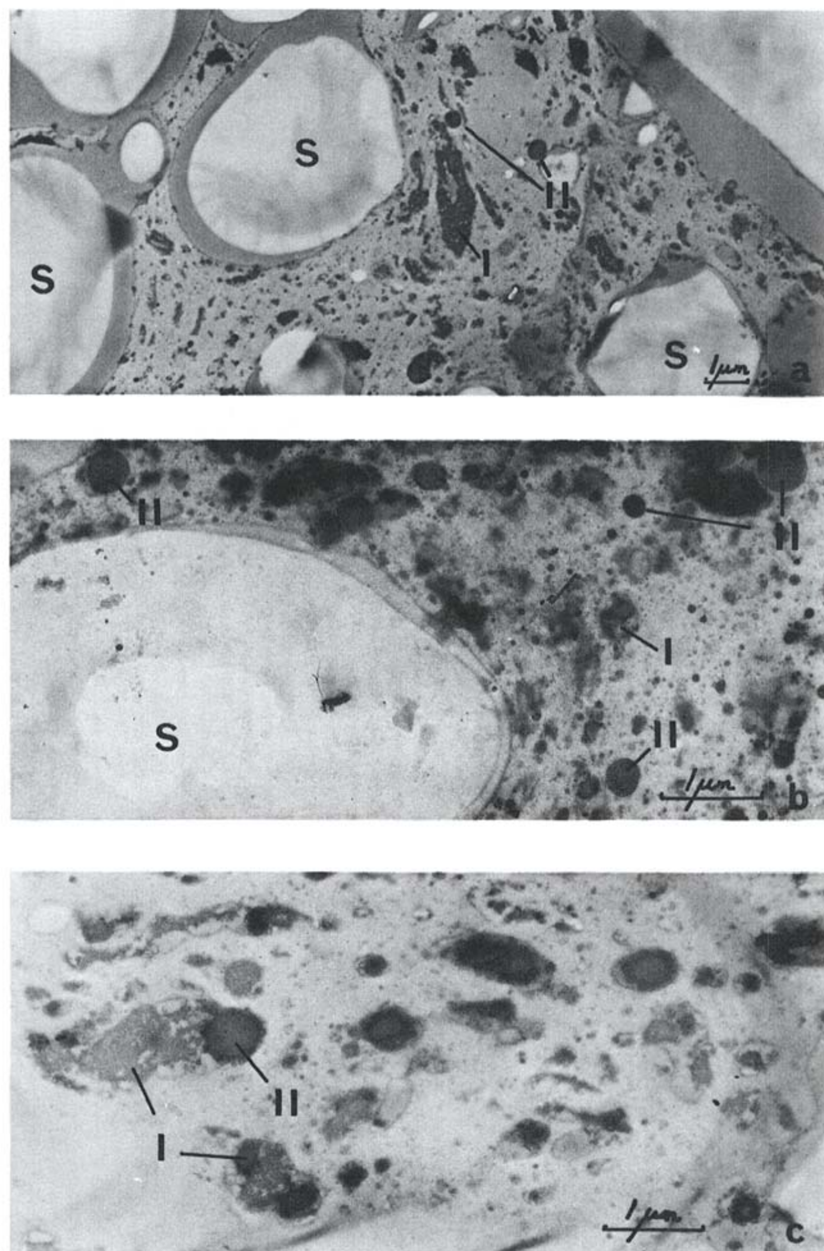

Fig. 1. Transmission electron micrographs of dough sections prepared from: a) Timgalen, b) Falcon, and c) Summit flours. S = Starch granules; I and II = Types I and II inclusions.

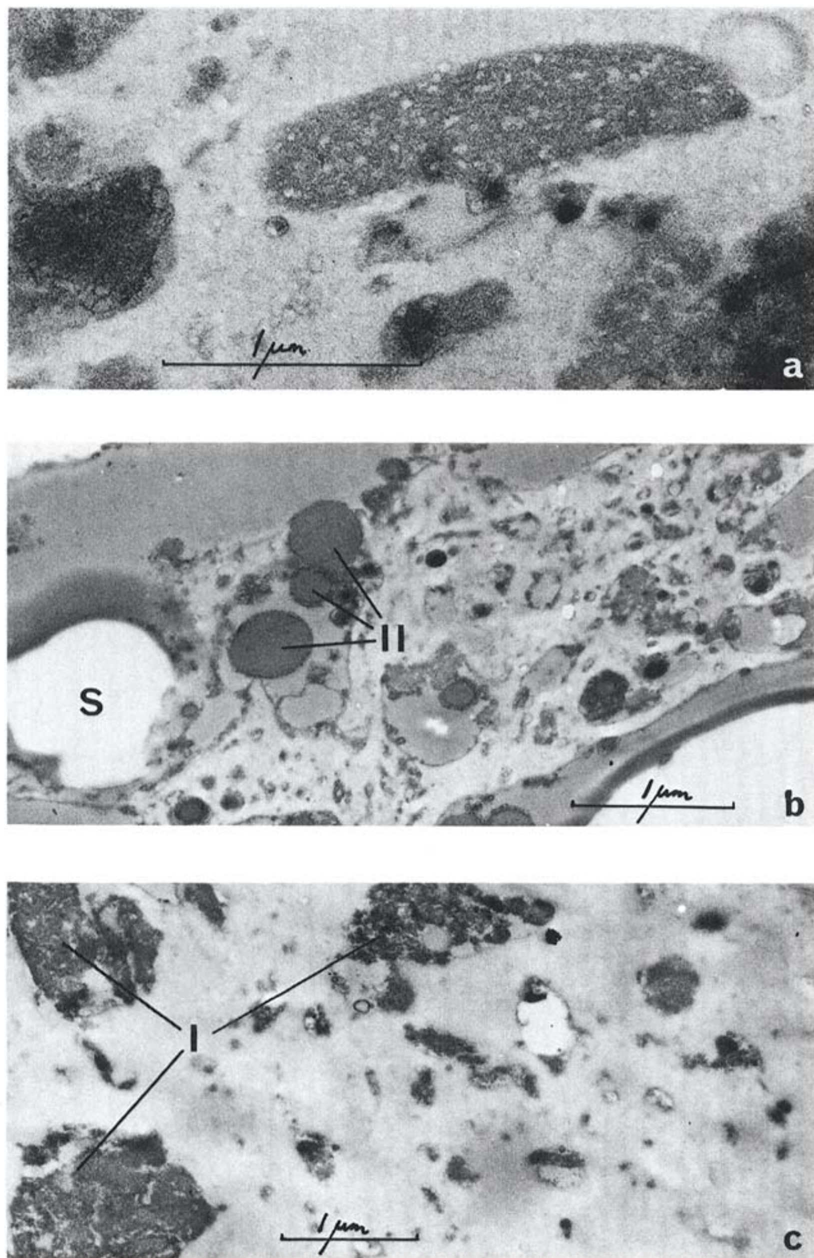

Fig. 2. Transmission electron micrographs of osmiophilic inclusions in dough. a) Type I inclusion at high magnification (48,000×). (From a dough prepared from Timgalen flour.) b) Normal New South Wales commercial flour illustrating Type II inclusions. c) The same flour after extraction with petroleum ether.

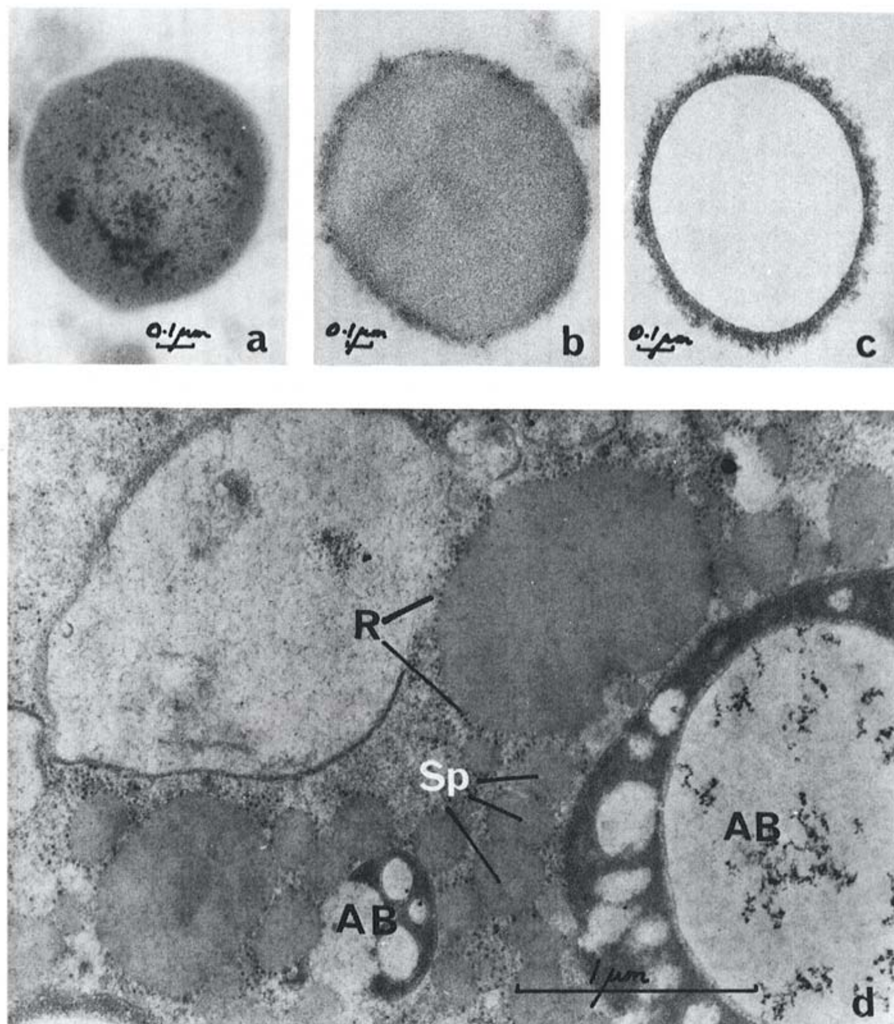

Fig. 3. Transmission electron micrographs of Type II osmiophilic inclusions in doughs derived from: a) Timgalen, b) Summit, and c) New Sough Wales commercial flours; and d) Lipid inclusions (spherosomes) in aleurone tissue. Sp = spherosomes; AB = aleurone bodies; and R = ribosomes.

possibility must also be considered that these lipid inclusions are derived from the spherosomes of aleurone and scutellar tissue, illustrated in Fig. 3, d. Seckinger and Wolf (25) report the presence of similar lipid droplets in flour samples spread on water and examined by electron microscopy.

Further evidence for the presence of interaction products derived from lipid and protein in the case of gluten, but not of storage protein, was obtained by microscopic examination of the urea-insoluble fraction which had been banded in a

sucrose density gradient. The very distinct differences between the two preparations are illustrated in Fig. 4, a and b.

The storage-protein preparation shows evidence of the presence of membrane residues and organelle remnants similar in structure to Type I inclusions in dough. Many of these particles retained sufficient ultrastructure to allow their recognition as rolled-up pieces of endoplasmic reticulum (Fig. 4, a, inset). Ultrasonication completely disintegrated these structures, yielding microscopically featureless protein in the banded fraction, together with a floating layer of lipid. The banded fraction prepared from gluten, on the other hand, consisted—in addition to badly degraded endoplasmic reticulum remnants—of osmiophilic vesicles of various types, as illustrated in Fig. 4, b. Similar vesicular structures also occur in dough (Fig. 2, b, arrowed). Also in the gluten preparation were numerous myelin figures of different types, some very similar to those illustrated by Zahler and Weibel (26). One such figure is shown in Fig. 4, b, inset.

Hydrolysate lipid analyses were carried out on the original storage protein and gluten preparations, as well as on the urea-soluble and -insoluble fractions derived from them. To obtain accurate results it was found necessary to dialyze each fraction free of urea and to freeze-dry them prior to analysis. The distribution of lipid between the two fractions is summarized in Table I.

Because of the quantities required for each hydrolysate lipid determination, the duplicate results quoted in Table I represent single analyses on samples prepared in replicate experiments. It will be seen from these results that in all cases the urea-insoluble fraction contained the highest proportion of lipid. Compared with gluten, storage protein is depleted of lipid during its preparation. This is reflected in the lower lipid content of the urea-insoluble fractions prepared from the latter. The extracted lipid can be replaced, leading to the production of urea-insoluble fractions comparatively high in lipids. That the lipids were firmly bound, and not merely associated with the insoluble fractions, is indicated by the fact that the lipid

TABLE I. LIPID DISTRIBUTION IN STORAGE PROTEIN AND GLUTEN, AND IN THE UREA-SOLUBLE AND UREA-INSOLUBLE FRACTIONS DERIVED FROM THEM

Sample	Hydrolysate Lipid (% dry weight)	
	Timgalen	Heron
Storage protein		
Original sample	1.4	1.3
Urea-soluble fraction	0.5	0.5
Urea-insoluble fraction	4.0;6.3	3.9;5.9
Storage protein mixed with water		
Urea-insoluble fraction	3.7;3.2	3.6;2.4
Storage protein mixed with water and lipid		
Urea-insoluble fraction	12.0;16.3	12.1;16.2
Gluten		
Original sample	3.4	3.0
Urea-soluble fraction	0.5	0.6
Urea-insoluble fraction	10.8;13.6	11.2;10.8

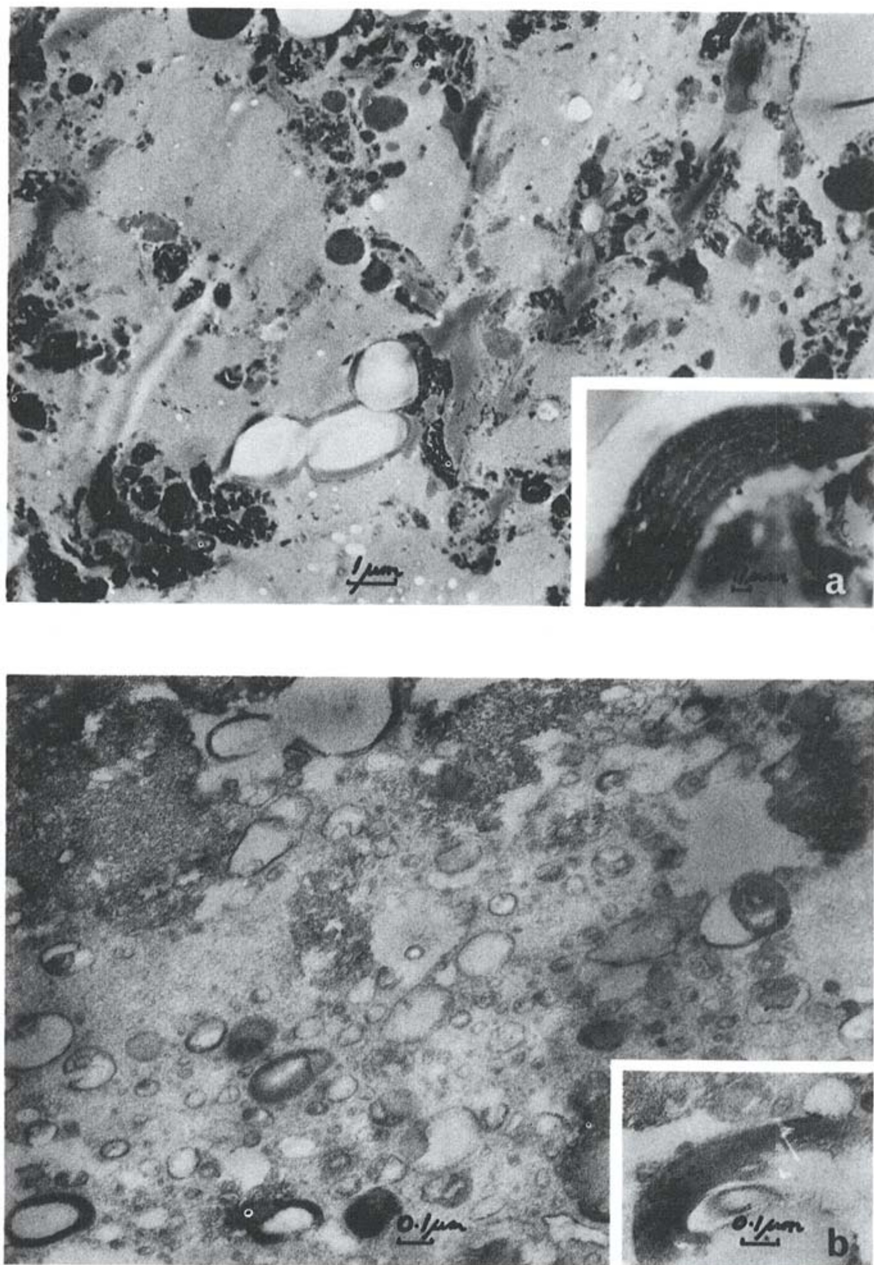

Fig. 4. Transmission electron micrographs of 4M urea-insoluble material from a) storage protein and b) gluten, suspended in a 20 to 60% sucrose density gradient. The fraction banding between 50 to 60% sucrose was examined. ER = endoplasmic reticulum remnant; VES = lipoprotein vesicles of various types; and MY = myelin figure from lipid-rich area.

could not be extracted from them with nonpolar solvents such as petroleum ether. In fact, prolonged extraction (48-hr.) with water-saturated *n*-butanol was necessary before sufficient lipid could be dissociated from them for thin-layer chromatography. The latter technique confirmed qualitatively that all the lipid components normally extracted from whole gluten by *n*-butanol were present in the urea-insoluble fraction.

GENERAL DISCUSSION

The unequivocal demonstration of endoplasmic reticulum and other membraneous residues in mature wheat endosperm cells (2) provides an immediate explanation of a major source of lipids in wheat flour, apart from any adventitious contribution from germ, aleurone, and scutellar tissue. Some of this lipid will be present as bound lipoprotein in the membrane structures themselves, whereas the remainder (sometimes described as "free" lipid) may be released from these structures during dough-mixing and gluten formation, to interact with other storage-protein components.

Transmission electron microscopy of dough reveals the presence of osmiophilic inclusions which appear to represent the membrane residues from which excess lipid has been removed. These are the Type I inclusions shown in Figs. 1 and 4. Any free excess lipid appears as discrete droplets (Type II inclusions, Figs. 1 and 3), whereas the remainder forms lipoprotein, which cannot be distinguished in the electron micrographs of dough, but which may be recognized by its characteristic appearance in the urea-insoluble fraction. Storage protein, as prepared by suspension in chloroform-benzene mixtures, is depleted of free lipid, and the urea-insoluble fraction of this material is comprised principally of membrane remnants. Interaction between any remaining lipid and protein is also prevented in this case by the presence of the urea.

The results presented in this and the preceding paper suggest that the extended gluten network in dough comprises the following protein, lipid, and lipoprotein sub-fractions:

a) Gliadin proteins of molecular weight less than 100,000. This is an arbitrary figure, since these form a strongly interacting system (27,28). These proteins are urea-soluble.

b) A urea-soluble, high-molecular-weight fraction present in the supernatant from the density-gradient separations. This material can be fractionated from gliadin by gel filtration, since its molecular weight is in excess of 100,000 (29), and appears to comprise two major sub-fractions (22).

c) Urea-insoluble membrane residues which have lost excess lipid (Type I osmiophilic inclusions).

d) Lipid-rich droplets carrying lipoprotein interaction products at their surface (Type II osmiophilic inclusions).

e) Urea-insoluble lipid-protein interaction products of high molecular weight formed during dough and gluten preparation. These are derived from the excess lipid (d, above) and certain storage-protein fractions, possibly the urea-soluble glutenin of medium and high molecular weights. As obtained by urea extraction and density-gradient centrifugation, this fraction appears as in Fig. 4, b.

Of the above sub-fractions, class a would be classified as gliadin, and classes b to e as glutenin, by currently accepted criteria.

The distribution within the original dough and gluten of class e glutenin, visualized under the electron microscope as small vesicles, 0.05 to 0.2 μm. in diameter, is open to speculation. By analogy with the behavior of endoplasmic reticulum in the intact cell, it might be expected to be present as an extensive network or reticulum, which collapses to form small vesicles (equivalent to microsomes) when the supporting proteins (gliadins and class b glutenins) are removed by extraction with urea. This hypothesis is illustrated in Fig. 5, a to d.

Grosskreutz (30) has proposed a model for gluten structure in which some 5% of the elastic sheet consists of a lipoprotein fraction which provides a slip plane

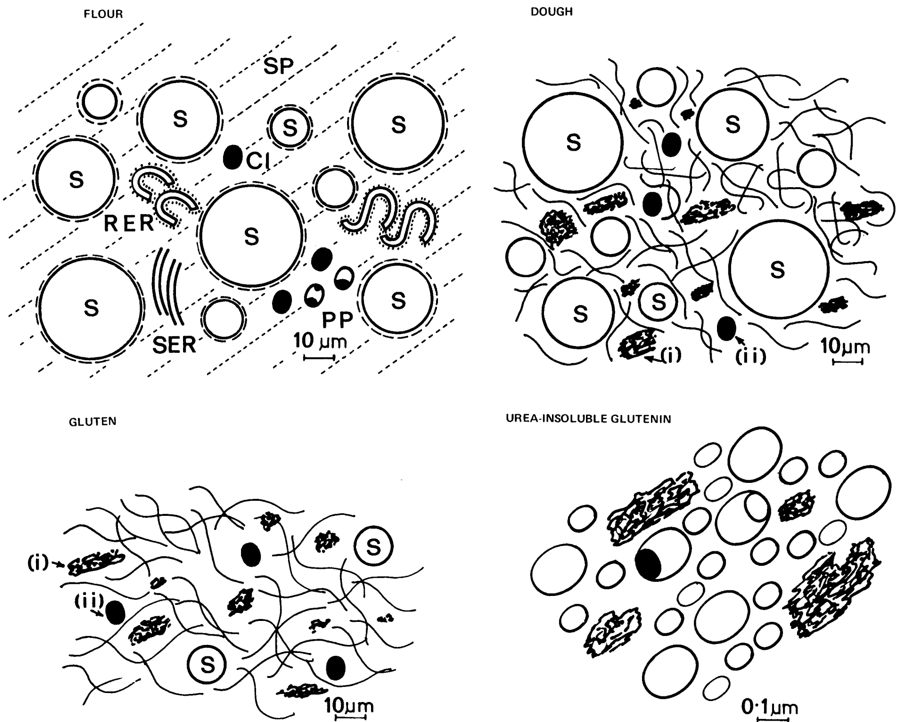

Fig. 5. Diagrammatic view of changes which occur in the conversion of storage protein to gluten, and in the formation and isolation of class e glutenin. See text for description.

Top left: Wheat flour, showing starch granules (S), storage protein (shaded background, SP), and inclusions: RER = rough endoplasmic reticulum; SER = smooth endoplasmic reticulum; PP = multi-vesicular bodies; and CI = osmiophilic cytoplasmic inclusions.

Top right: Dough, showing gliadin and lipoprotein network surrounding starch granules, and Type I and Type II inclusions.

Lower left: Gluten, from which substantially all starch has been removed.

Lower right: Glutenin, from which all gliadin and urea-soluble (class b) glutenin molecules have been removed by extraction.

between adjacent protein platelets. While agreeing that the lipoprotein fraction in question accounts for some 5 to 10% of gluten, it is felt that the structures revealed by the present investigations do not support this model. There appears to be no evidence for slippage within the lipid-protein interaction products themselves, nor do the discrete lipid inclusions (class d) lend themselves to this role. As illustrated in Fig. 5, b, this lipoprotein fraction is believed to form an extended, interwoven network in which interchain entanglement and slippage would provide a molecular explanation of the rheological phenomena involved.

If this is true, this fraction may be expected to play a most important role in determining dough structure and rheological behavior.

Acknowledgements

It is a pleasure to acknowledge the assistance of Miss K. K. Barlow in this work. Hydrolysate lipid analyses were kindly carried out by F. MacRitchie and P. W. Gras. The author is indebted to M. Vesk, Sydney University Electron Microscopy Unit, for preparation of the electron micrographs.

Literature Cited

1. SECKINGER, H. L., and WOLF, M. J. Lipid distribution in the protein matrix of wheat endosperm as observed by electron microscopy. Cereal Chem. 44: 669 (1967).
2. SIMMONDS, D. H. The ultrastructure of the mature wheat endosperm. Cereal Chem. 49: 212 (1972).
3. SIMMONDS, D. H. Morphological and molecular aspects of wheat quality. Wallerstein Lab. 34: 17 (1971).
4. BUTTROSE, M. S. Ultrastructure of the developing wheat endosperm. Aust. J. Biol. Sci. 16: 305 (1963).
5. JONES, R. L. The fine structure of barley aleurone cells. Planta 85: 359 (1969).
6. SWIFT, J. G. Structure and cytology of the wheat scutellum. M. Sc. Thesis submitted to Monash University, Clayton, Victoria, Australia.
7. McCAIG, J. D., and McCALLA, A. G. Changes in the physical properties of gluten with aging of flour. Can. J. Res. Sect. C. 19: 163 (1941).
8. OLCOTT, H. S., and MECHAM, D. K. Characterization of wheat gluten. I. Protein-lipid complex formation during doughing of flours. Lipoprotein nature of the glutenin fraction. Cereal Chem. 24: 407 (1947).
9. MECHAM, D. K., and WEINSTEIN, N. E. Lipid binding in doughs. Effects of dough ingredients. Cereal Chem. 29: 448 (1952).
10. CHIU, C-M, and POMERANZ, Y. Changes in extractability of lipids during bread-making. J. Food Sci. 31: 753 (1966).
11. DANIELS, N. W. R., RICHMOND, J. W., RUSSELL EGGITT, P. W., and COPPOCK, J. B. M. Studies on the lipids of flour. III. Lipid binding in breadmaking. J. Sci. Food Agr. 17: 20 (1966).
12. DANIELS, N. W. R., RICHMOND, J. W., RUSSELL EGGITT, P. W., and COPPOCK, J. B. M. Effect of air on lipid binding in mechanically developed dough. Chem. Ind. (London) 1967, p. 955.
13. DANIELS, N. W. R., RICHMOND, J. W., RUSSELL EGGITT, P. W., and COPPOCK, J. B. M. Studies on the lipids of flour. IV. Factors affecting lipid binding in breadmaking. J. Sci. Food Agr. 20: 129 (1969).
14. BLISH, M. J. The wheat flour proteins. Cereal Chem. (Suppl.) 13: 16 (1937).
15. BOTTOMLEY, R. A., McAUSLAN, B. R., and POWELL, A. D. G. Lipid-protein complex of wheat flour. Chem. Ind. (London) 1958, p. 1476.
16. ZENTNER, H. The continuous electrophoresis of wheat gluten. Chem. Ind. (London) 1960, p. 317.

17. ROHRLICH, M., and NIEDERAUER, T. Fat-protein complexes in cereals. II. Composition of the protein and lipid components. Fette Seifen Anstrichm. 70: 58 (1968). [Chem. Abstr. 68: 86195f (1968).]
18. FISHER, N. Wheat lipids. In: Recent advances in food science, ed. by J. Hawthorn and M. J. Leitch; vol. 1, p. 226. Butterworths: London (1962).
19. LEE, C. C., and WAN, K-M. Studies with radioactive tracers. VII. Investigations with flour containing P^{32}. Cereal Chem. 40: 415 (1963).
20. PONTE, J. G., Jr., De STEFANIS, V. A., and COTTON, R. H. Studies of gluten lipids. I. Distribution of lipids in gluten fractions separated by solubility in 70% ethanol. Cereal Chem. 44: 427 (1967).
21. ROUSER, G., NELSON, G. J., and FLEISCHER, S. In: Biological membranes: Physical fact and function, ed. by D. Chapman, p. 37. Academic Press: New York (1968).
22. SIMMONDS, D. H., and WRIGLEY, C. W. The effect of lipid on the solubility and molecular-weight range of wheat gluten and storage proteins. Cereal Chem. 49: 317 (1972).
23. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. Official methods of analysis, 10th ed., p. 194. The Association: Washington, D.C. (1965).
24. GREENWOOD, A. D., LEECH, RACHEL M., and WILLIAMS, J. P. The osmiophilic globules of chloroplasts. Biochim. Biophys. Acta 78: 148 (1963).
25. SECKINGER, H. L., and WOLF, M. J. Electron microscopy of endosperm protein from hard and soft wheats. Cereal Chem. 47: 236 (1970).
26. ZAHLER, P., and WEIBEL, E. R. Reconstitution of membranes by recombining proteins and lipids derived from erythrocyte stroma. Biochim. Biophys. Acta 219: 320 (1970).
27. WINZOR, D. J. Detection of interaction in gluten extracts by gel filtration. Arch. Biochem. Biophys. 113: 421 (1966).
28. RONALDS, J. A., and WINZOR, D. J. Partial characterization of interactions in aqueous gluten extracts. Arch. Biochem. Biophys. 129: 456 (1969).
29. BUSHUK, W., and WRIGLEY, C. W. Glutenin in developing wheat grain. Cereal Chem. 48: 448 (1971).
30. GROSSKREUTZ, J. C. A lipoprotein model of wheat gluten structure. Cereal Chem. 38: 336 (1961).

[Received August 10, 1971. Accepted December 29, 1971]