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# MICROSTRUCTURAL STUDIES OF GLUTEN AND A HYPOTHESIS ON DOUGH FORMATION

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## Abstract

The transformation of a mixture of water and wheat flour into a viscoelastic dough was studied by light microscopy, scanning and transmission electron microscopy. In flour particles, gluten protein fills up the space between the starch granules forming a three-dimensional network. Dough formation is the result of the adhesion of the protein networks of individual flour particles to one another and their extension during kneading. In the course of the kneading process protein films form from the strands by bidirectional extension. The strands of the gluten network consist of randomly arranged protein threads which are twisted to form loops and knots in the unextended state. Bidirectional extension of the gluten pulls apart these knots to platelet-like forms.

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**Key words**: Flour particles, dough formation, gluten formation, light microscopy, scanning and transmission electron microscopy.

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## Introduction

Wheat endosperm proteins possess the unique property of forming gluten when mixed with water. Although the process of dough and gluten formation is under thorough investigation by chemical and rheological methods, it is not yet completely understood.Relatively limited information is available concerning the microstructural alterations of endosperm protein during dough and gluten formation. Microscopic studies (Bechtel et al., 1982; Bechtel and Barnett, 1986) have shown that during development of the wheat kernel, protein granules form within the endosperm cells and fuse to form a protein matrix. Moss (1972) used light microscopy to study dough formation. In an undermixed dough, he observed compact protein masses stretched out into sheets during mixing. Sheets as well as fibrils are reported by other authors to be elements of dough (Bechtel et al., 1978; Fretzdorff et al., 1982; Paredes-Lopez and Bushuk, 1983). Grosskreutz (1961) investigated gluten by transmission electron microscopy and small angle X-ray scattering and reported a layerlike arrangement of protein sheets in stretched gluten membranes. These sheets were found to be composed of protein platelets.

Only recently, the structure of gluten protein has been observed in flour particles as well as in dough and in gluten at magnifications up to the macromolecular level (Amend and Belitz, 1989, 1990a-c). In this paper, these results and some new findings are combined and integrated into a diagram to demonstrate the changes in the microscopic appearance of the gluten protein at various stages of dough formation. Based on these observations, a hypothesis is put forward on the process of dough formation.

## Materials and Methods

Although the details of the preparation procedures have already been published (Amend and Belitz, 1989, 1990a-c; Amend, 1990), a brief description is given, emphasizing the more significant steps within the procedures.

All investigations were made using the German winter wheat variety Rektor which is a strong bread-making wheat. Dough was prepared with distilled water in a Brabender farinograph; gluten was washed out in a glutomatic gluten washer (Falling Number, Huddinge, Sweden). Free protein (wedge protein) was prepared as described by Amend and Belitz (1990a).

For light microscopy (LM), flour particles were placed on a slide, a cover glass was added and water was introduced between the glasses. The particles were stretched by manually sliding the cover glass across the slide. To spread flour particles at the air/water interface, a drop of water, or of an aqueous solution of dithioerythritol (1%, w/v), was placed on a slide and a few flour particles were sprinkled on it. Observations at the microscope (Zetopan, Reichert, Vienna) were made using phase contrast and asymmetric illumination contrast (Kachar, 1985) without any stain at magnifications between 40x and 320x. The observations were recorded using a video-camera attached to the microscope. Micrographs were taken from the screen.

For scanning electron microscopy (SEM), hydrated samples of dough, gluten and single flour particles were either critical-point-dried (CPD) after glutaraldehyde fixation, or freeze-dried. Starch was removed by treatment with a solution of 0.05 % α-amylase (Bacillus subtilis) for 1 h at room temperature, after holding the sample at 85°C for a few seconds. Pieces of dough and gluten were stretched into membranes and washed thoroughly in distilled water (Millipore MilliQ-quality) prior to fixation. After sputtering with gold, the samples were observed in a Jeol JSM 35-C scanning electron microscope. For scanning transmission electron microscopy (STEM), single hydrated flour particles, still containing the starch, were shadowed with tantalum/tungsten (3 nm). The samples were observed by using a Jeol JEM 2000-FX microscope. Further details of the preparations are presented elsewhere (Amend and Belitz, 1990 b; Amend, 1990).

Except as noted otherwise, samples for transmission electron microscopy (TEM) were produced by a bare-grid-preparation. In addition to dough and wet gluten, single flour particles and wedge protein, termed 'free' protein according to the nomenclature of Rohrlich et al. (1972), were also used for fine structure investigations. Some of the specimens were treated with an aqueous solution of dithioerythritol (DTE, 1 %, w/v) or triethylamine (TEA, 1 %, v/v). The specimens for TEM were critical-point-dried or freeze-dried (freeze-dried specimens are identified in figure captions). To reduce freezing artifacts, specimen thickness during freezing by the double-sided propane jet method did not exceed 20 µm. The samples were shadowed with tantalum/tungsten and observed at a Jeol-100CX TEM. For further details see Amend and Belitz (1990 c) and Amend (1990). Visible alterations in the protein structure of specimens treated with α-amylase due to possible proteolytic side-activities were not observed when compared to untreated samples. Starch was digested in samples used for SEM investigation only.

## Results

### Observations on Single Flour Particles

The light microscope offers the unique opportunity to observe biological material without any of the pretreatment which is usually necessary for electron microscopy. The insoluble endosperm protein of flour particles can be well observed without the use of any staining. This is an important precondition for studying the behaviour of the protein under mechanical stress. Single, hydrated flour particles can be manipulated easily while being observed, as they stick to both the slide and the cover glass. The flour particles were extended by shifting the cover glass by hand. On unextended hydrated flour particles, the structure

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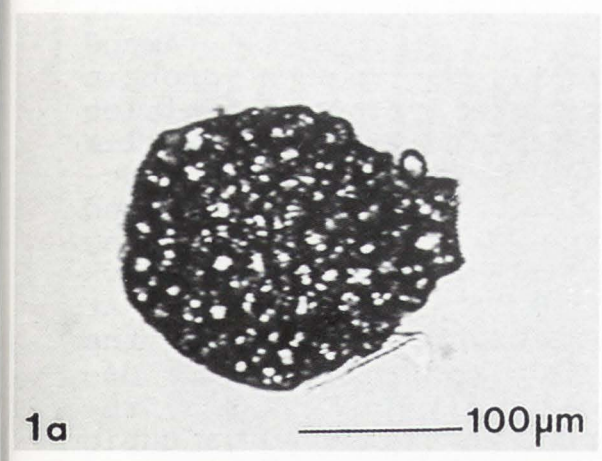
*Fig. 1-5: Light micrographs, Fig. 6-8: SEM-micrographs.*
*Fig. 1. a: Single flour particle in water, b: flour particle slightly extended by shifting the cover glass, c: strongly extended flour particle.*
*Fig. 2. Extended protein strands sticking to the slide at one end.*
*Fig. 3. Network of protein strands after bidirectional extension of a flour particle.*
*Fig. 4. Protein film extending between curved strands (arrowed).*
*Fig. 5. Flour particle spread at the water surface. Curved strands (arrowed) indicate protein films that are streched out between them.*
*Fig. 6. Unextended flour particle with the starch removed enzymatically. The holes mark the positions of big and small starch granules.*
*Fig. 7. Extended flour particle (starch removed).*
*Fig. 8. Bidirectionally extended flour particle (starch removed). Between the strands, protein films are stretched out.*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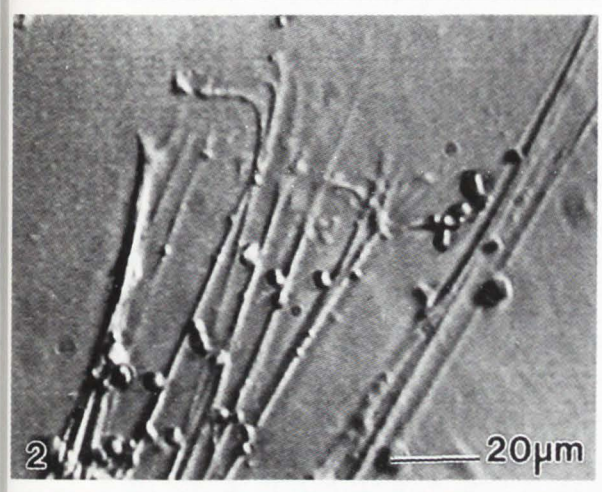
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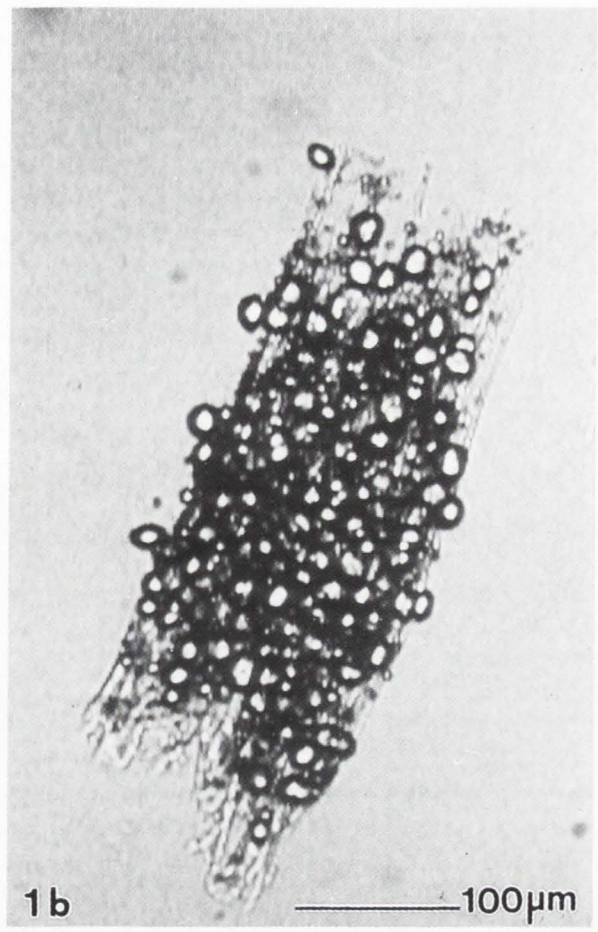
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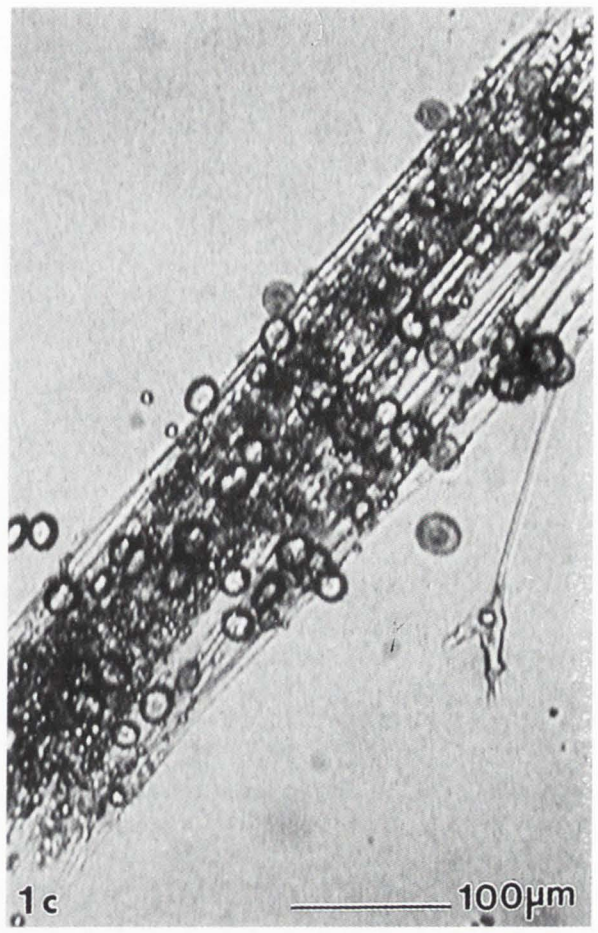
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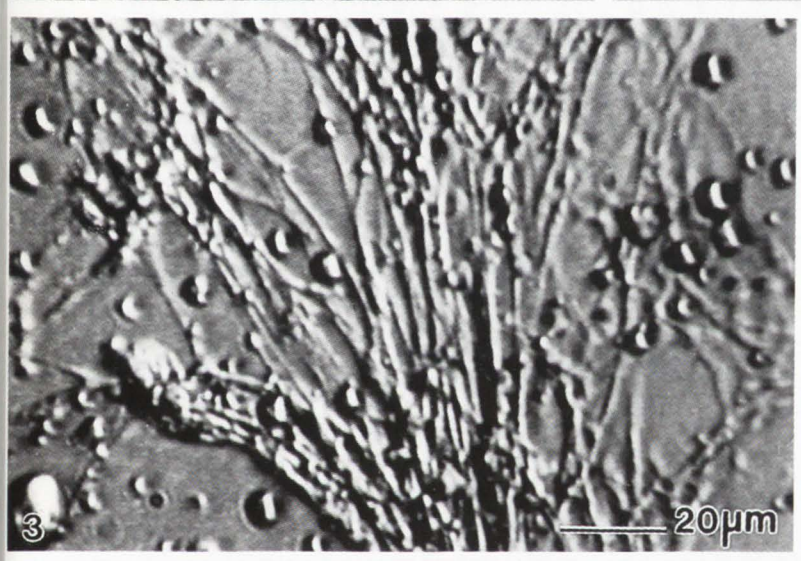
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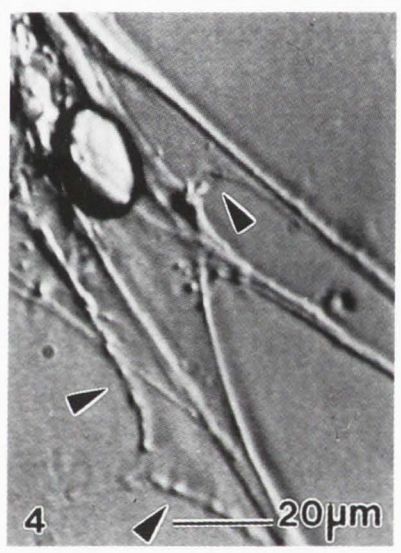
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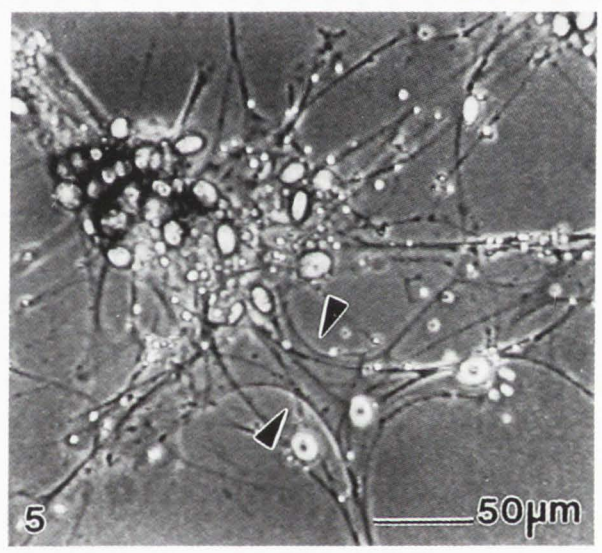
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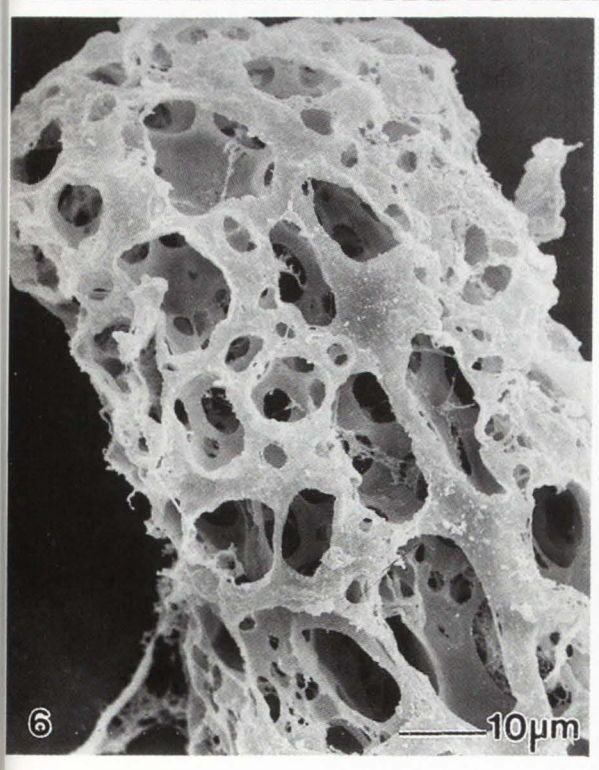
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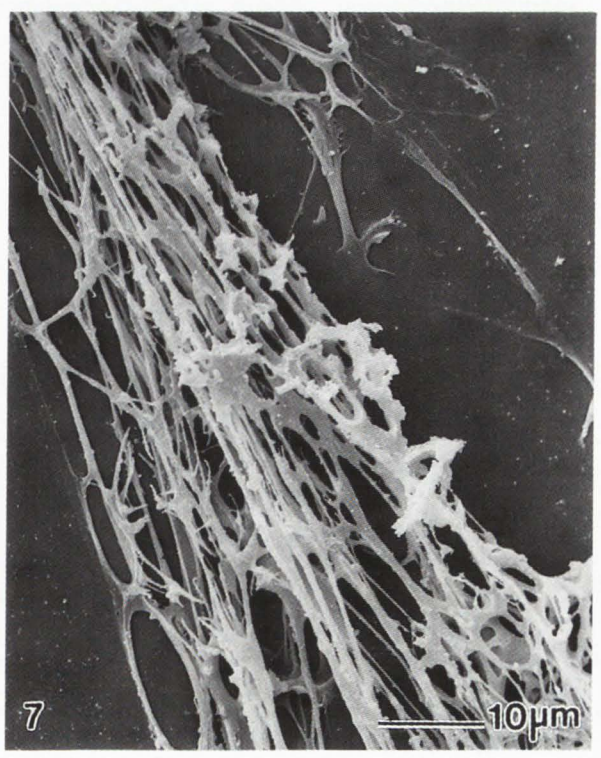
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of the protein was barely discernible (Fig. 1a). Extending a flour particle unidirectionally revealed numerous strands that were oriented in the direction of stress (Figs. 1b,c). Stretched strands, sticking to the glass slide at one end can be seen in Fig. 2. If the cover glass was then moved sideways, the particle was torn apart bidirectionally (Fig. 3), releasing most of the starch granules. Films were formed between the strands which therefore became evenly curved (Fig. 4). The strands could be aggregated easily into a ball by moving the coverglass circularly. The ball, as well as the strands corresponded to gluten in amino acid composition (Amend and Belitz, 1989). When the ball touched neighbouring hydrated flour particles, their protein strands adhered to the gluten ball and increased it in size. A different way to extend a flour particle is by spreading it on a water surface. Due to the surface tension, the particle is torn apart bidirectionally at the interface immediately after water contact (Bernardin and Kasarda, 1973; Amend and Belitz, 1989, 1990a). Interconnected protein strands appeared (Fig. 5) with protein films stretched between the curved strands. If flour particles were spread on a solution of the reducing agent dithioerythritol, strands appeared initially but after some seconds they disrupted and retracted slowly.

The gluten protein network can also be prepared for observation by SEM. To investigate its original state in unextended hydrated flour particles, the starch was removed enzymatically. The remaining gluten filled up the space between the starch granules and appeared sponge-like or network-like (Fig. 6), depending on the amount of protein, which is higher at the periphery of the wheat kernel than at its center (Simmonds, 1974). A unidirectionally extended flour particle can be seen in Fig. 7 with the strands of the gluten network stretched out (starch digested). Bidirectional extension formed protein films from the strands (Fig. 8, starch digested) which are similar to the films of Figs. 4 and 5. All structures, observed with extended single flour particles, could also be found in dough.

**Dough Formation**

Dough was investigated at different stages of development by SEM, STEM and TEM. The results are summarized schematically in a diagram (Fig. 9) to which the micrographs will be related. In Fig. 9, dough formation, i.e., kneading, proceeds vertically in three steps and the magnification within each step increases horizontally. The scale bars for the columns are indicated in the last line of the diagram.

**Aggregation of Flour Particles**. As demonstrated by light microscopy (Amend and Belitz, 1989), the gluten protein networks of hydrated flour particles stick together when individual particles come into contact. A similar process occured in dough after water was added and distributed in the flour at the beginning of kneading creating a continuous network structure (Fig. 10, starch digested). At this point, the network structure of the dough was mostly unextended. This is demonstrated schematically in Fig. 9 a, representing the protein network of a cubic section from a dough at the beginning of kneading. On the diagram, the starch is not shown, and cell wall residues that still exist in flour particles as envelopes around endosperm cells at the beginning of kneading are not considered. The branchings of the strands of the unextended network can be seen in Fig. 11 and are demonstrated schematically with three strands in the diagram (Fig. 9 b). Figs. 11 and 6 are rather similar, because at the beginning of kneading the flour particles in a dough are still unextended.

**Extension of the Protein Network**. At the beginning of the kneading process, the agglomeration of the flour particles was promoted by pressing them together intimately. In this way the kneading forces could be transmitted throughout the whole structure. In the shear field formed by the kneading process, the network structure was extended bidirectionally (Fig. 9e, Fig. 12) with the surface area getting larger and the depth smaller. Starch granules, trapped within the unextended network were liberated by the extension. At the branching points of the strands, the drag began to create films (Fig. 9f, Fig. 13).

**Film Formation**. Kneading the dough to optimal consistency further extended the protein network bidirectionally (Fig. 9i, 14). The strong bidirectional extension enlarged the films that began even to perforate (Fig. 9k, 15). The coarse structure of the underkneaded dough does transform into a more homogenous one, as a great number of strands transformed into films. The three-dimensional branching of the unextended network (Fig. 9a) still existed in the developed dough despite a strong orientation of the strands in the plane of stretching (Fig. 9i).

Gluten, washed out from dough, exhibited structures that were comparable to that of the gluten in dough. Gluten, stretched out into a membrane consisted of protein films in a sheet-like arrangement (Fig. 16). Heavy stretching perforated the gluten films to form a closely interconnected network of fine gluten fibrils (Fig. 17). When extended unidirectionally, however, gluten structure is dominated by strands rather than by films.

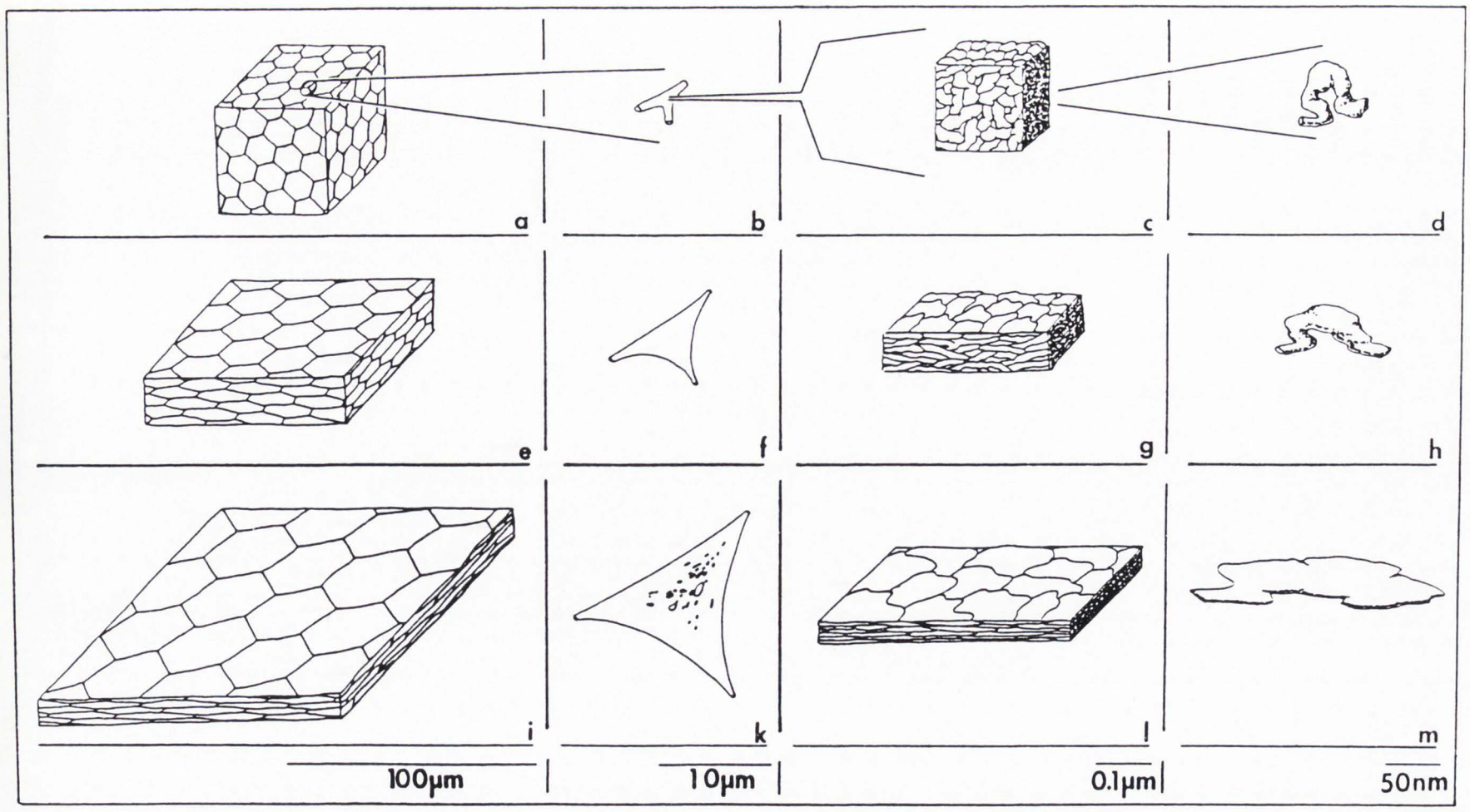
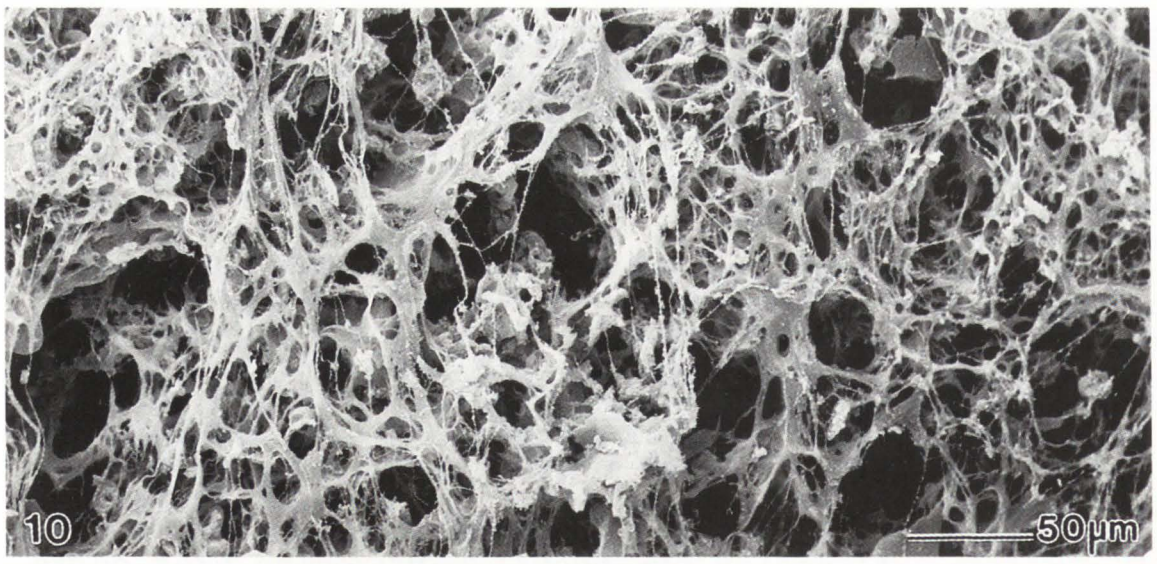



*Fig. 9. Schematical view of dough formation. The magnification increases horizontally, the kneading process proceeds vertically. The scale bars at the last line indicate the magnification for each column .*
*First column (Figs. 9a,e,i): Bidirectional extension of the three-dimensional gluten network of dough by kneading (spacial branching of the strands and starch granules not shown). The strands become oriented in the plane of stretch.*
*Second column (Figs. 9b,f,k): The branching point of three strands of the gluten network is extended bidirectionally to form a film which perforates due to the high extension (Fig. 9k).*
*Third column (Figs. 9c,g,l): Fine structure of a cubic section from a protein strand at different states of extension. Irregular globular surface structures in the unextended state (Fig. 9c) flatten down at high tension (Figs. 9g,l). The protein threads become oriented in the plane of stretch.*
*Fourth column (Figs. 9d,h,m): A piece of a single protein thread (Fig. 9d) as it may exist within the structures of the third column. It orients in the place of extension, flattens down and is finally torn apart bidirectionally. (Figs. 9h,m).*

## Fine Structures

The resolution power of the SEM is usually insufficient to observe the fine structures of the gluten strands or of the films. Therefore strands and films were observed at different states of extension by the STEM and the TEM at a macromolecular level of magnification. Due to thorough washing during the preparation of protein samples for TEM, the gliadin was partly removed. Therefore the remaining protein can be regarded as glutenin enriched gluten (Amend and Belitz, 1990c). For preparative reasons, fragments of the protein matrix, the wedge protein, were also used for this investigation. Unextended strands exhibited irregular globules at the surface (Fig. 18). For reasons to be discussed later, the globules were interpreted as loops and knots formed from protein threads which were strongly twisted and coiled up intestine-like (Fig. 9c). Bidirectional expansion of the strands (Fig. 9b, f, k) tore apart the globular surface structures and flattened them down (Fig. 9g, 19). Finally, the globules were extended into irregularly shaped platelet-like forms (Fig. 91, Fig. 20) which were arranged parallel to the plane of stretching. The thickness of these platelets was 10 nm or less. To demonstrate the effect of dough formation on the threads mentioned above, the transformations were shown with a piece of a hypothetical thread in the righthand column of the diagram in Fig. 9. In Fig. 9d, the thread was still relaxed, coiled up and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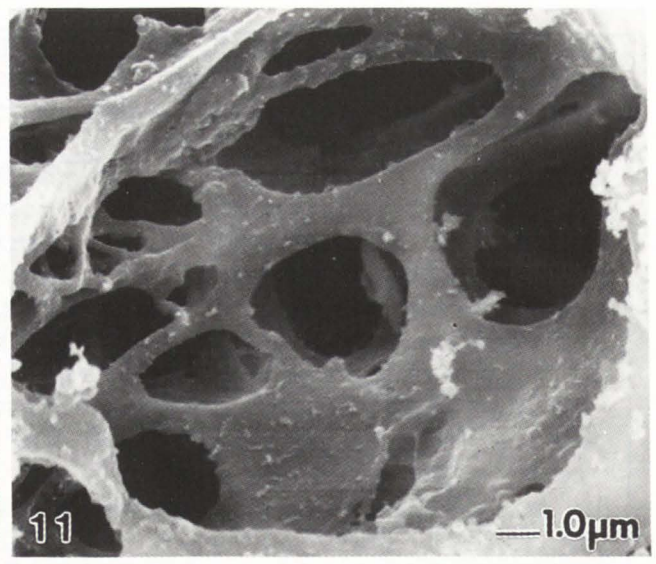
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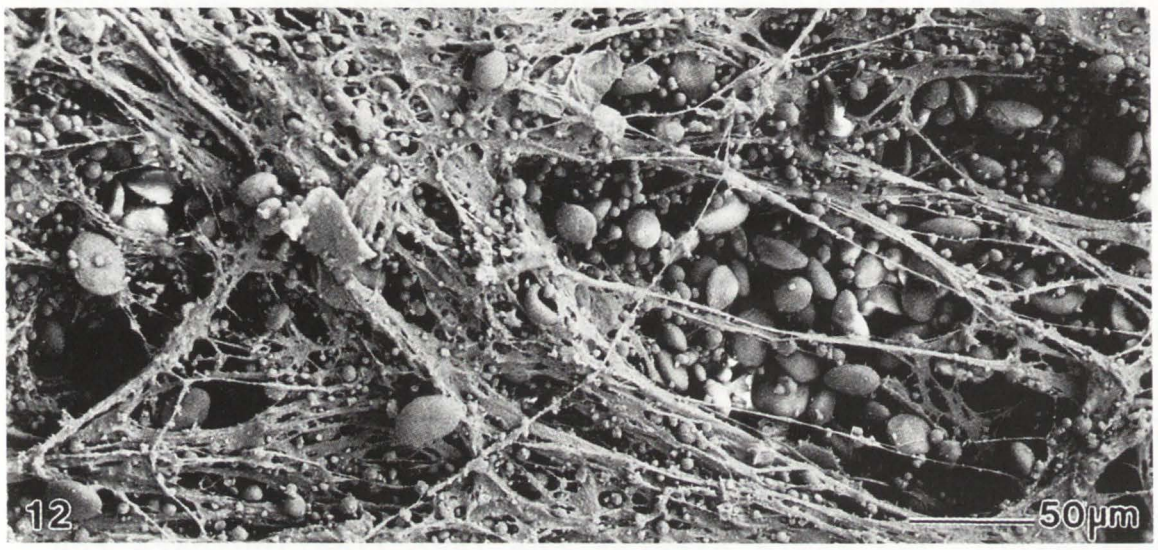
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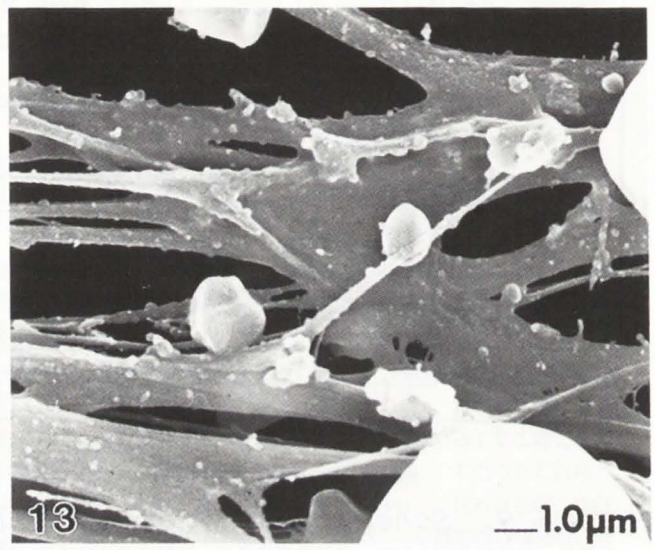
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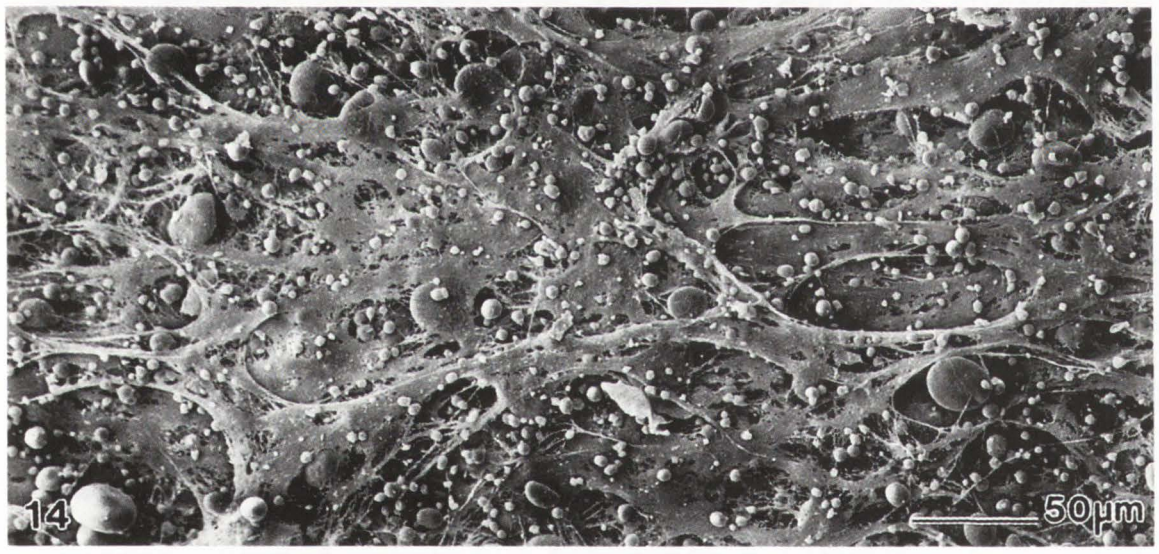
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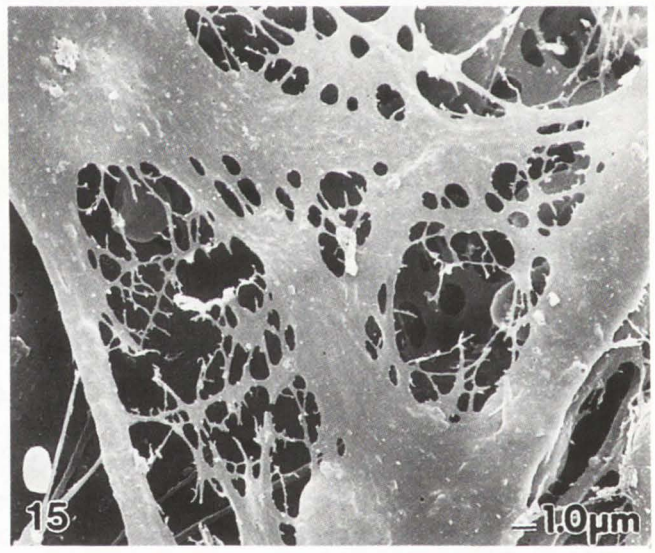
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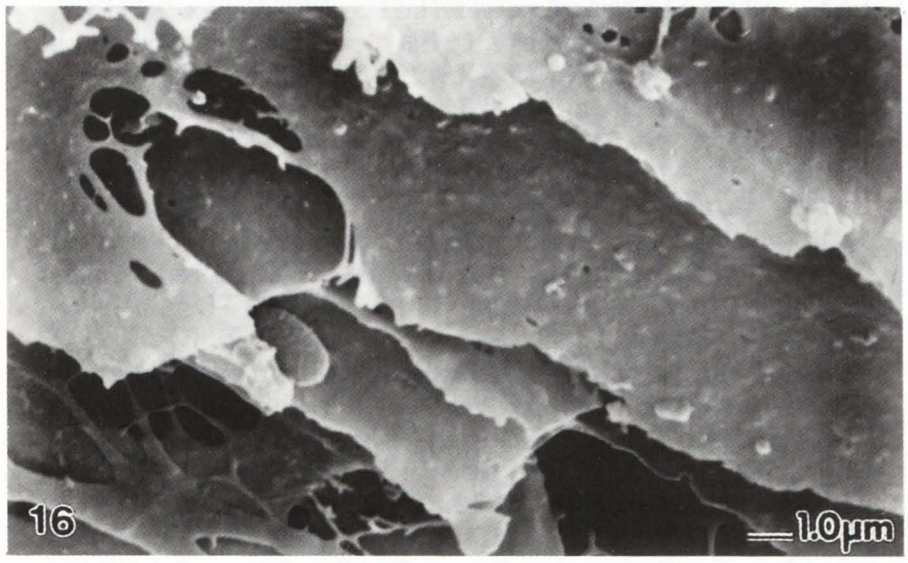
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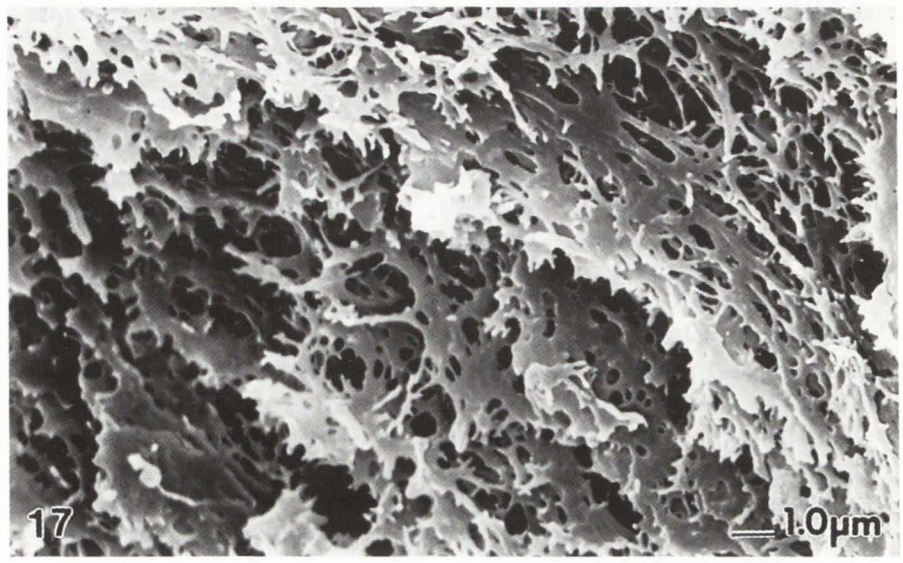
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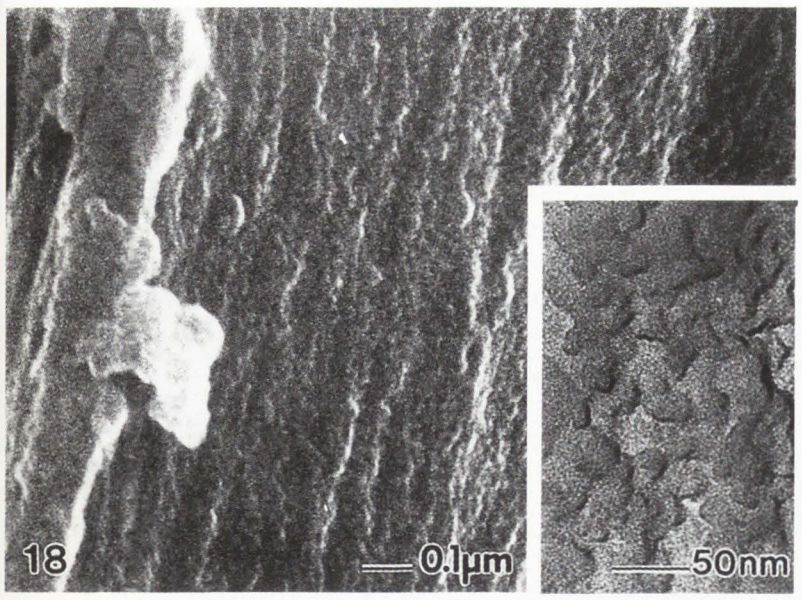


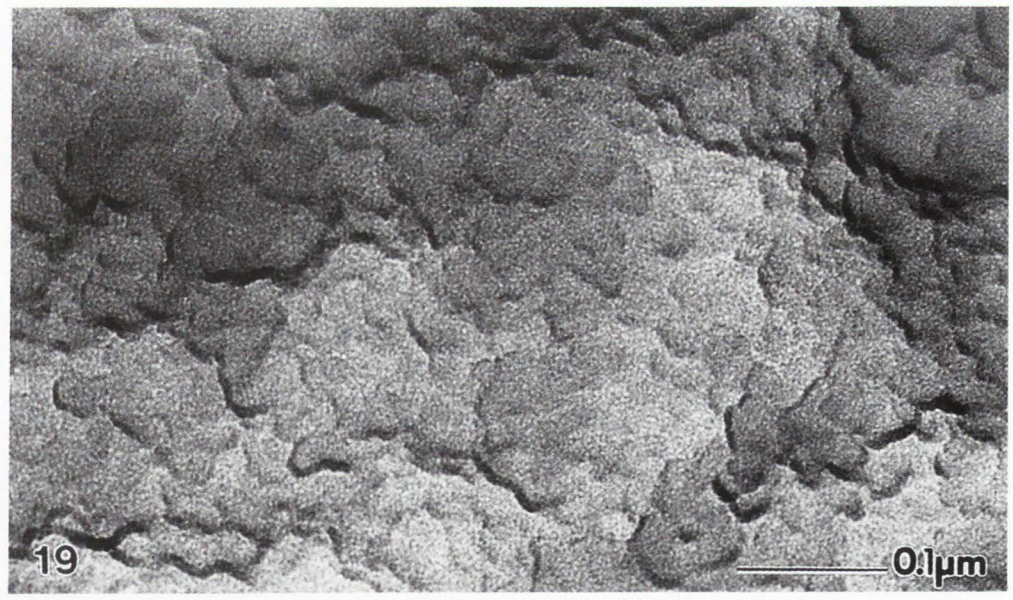


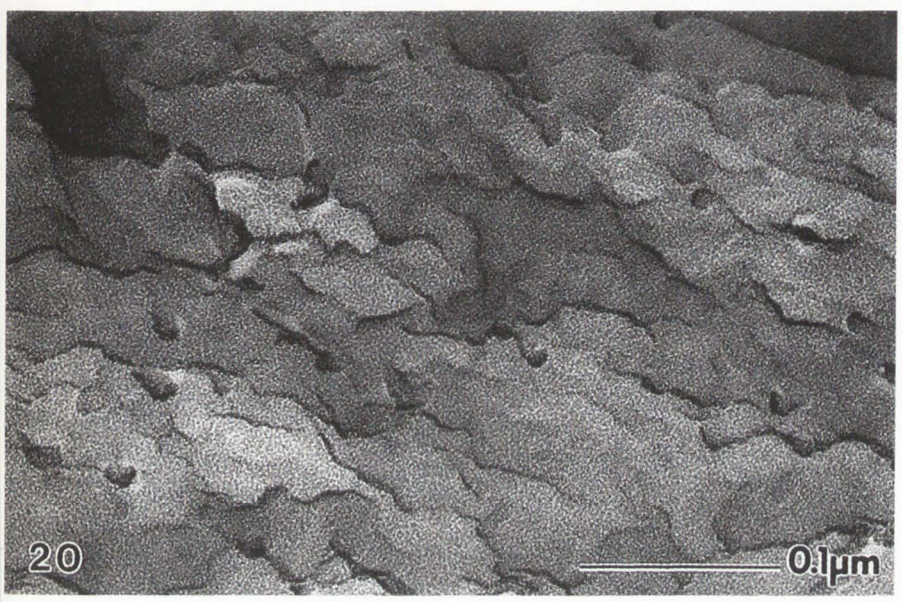


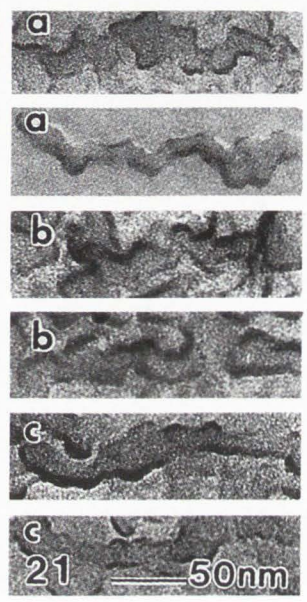


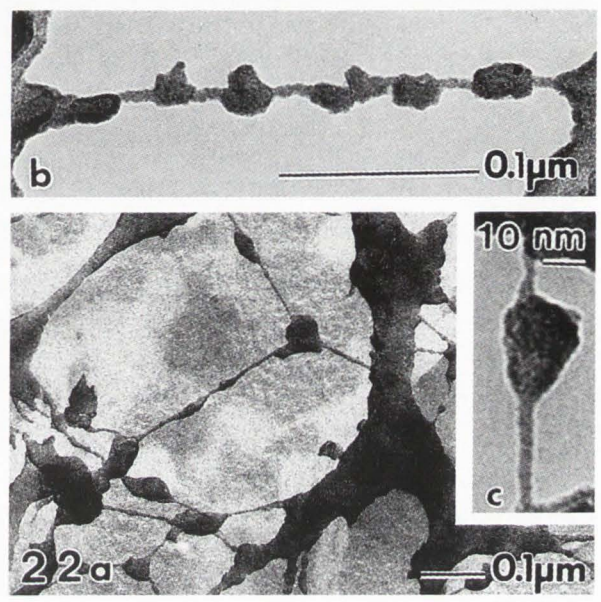


*Fig. 18-22: TEM-micrographs.*

*Fig. 18. Slightly extended gluten strand exhibiting a rough surface (STEM-micrograph). Inset: same structure at the TEM at higher magnification.*

*Fig. 19. Surface of an extended gluten film. Surface structures flatten down.*

*Fig. 20. Platelet-like forms of a strongly extended gluten film.*

*Fig. 21. Protein threads on gluten films in a) water (lower figure of a was freeze-dried), b) aqueous triethylamine (both figures freeze-dried) and c) aqueous dithioerythritol.*

*Fig. 22. a: Highly extended protein fibrils which show thickenings. Sample was freeze-dried and prepared on a formvar-coated grid. b, c: Fibrils at higher magnifications (CPD).*

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*Fig. 10-17: SEM-micrographs*

*Fig. 10. Dough after adding water; not kneaded (starch removed). Flour particles adhere together but network structure is still mostly unextended.*

*Fig. 11. Detail of the network structure of a flour particle in a dough that was not kneaded (starch removed).*

*Fig. 12. Dough with the network structure extended by kneading, but not fully developed.*

*Fig. 13. Detail of an extended network structure. Between the strands, protein films develop.*

*Fig. 14. Dough optimally kneaded. Protein films have formed due to bidirectional extension.*

*Fig. 15. Detail of bidirectionally extended network in dough. The protein film has partly perforated.*

*Fig. 16. Fracture face of a gluten membrane with protein films in a sheetlike arrangement.*

*Fig. 17. Heavily stretched gluten membrane with perforated protein films.*

without any orientation. In the course of expansion of the gluten film, the thread was oriented in the plane of stretching, and simultaneously, it was extended bidirectionally and flattened down (Fig. 9h,m). During unidirectional extension of protein strands, the threads were stretched out straight without forming platelets and became aligned in the direction of stretch.

**Protein Threads**. As discussed above, prior to extension, the threads exist in a strongly coiled form. This is based on observations of low extended protein films exhibiting some threads at the surface which had not been torn apart bidirectionally into platelets. Platelets of extended gluten films, upon treatment with TEA or DTE, retracted back to threads which remained unraveled. Despite the treatments, the films remained intact because, in contrast to the air/water-interface, the structures were not exposed to mechanical forces after the treatment (Amend and Belitz, 1990 c). Despite different treatments and preparation procedures, the threads looked similar. The diameter of the threads found in water, as well as after TEA or DTE treatment was about 10 to 30 nm, the total length and possible branching however is not yet known. Fig. 21 shows two threads each found in water and after treatment with TEA and DTE. The thinnest structures found were fibrils within highly perforated protein sheets. These fibrils sometimes were as thin as 3 nm including the shadowing material and some of them showed thickenings (Fig. 22).

## Discussion

### Artifacts

Dough and gluten is a complex material containing substances with different solubility properties and with a structure that is highly susceptible to mechanical influences. These factors largely contribute to the difficulties in the preparation of those materials. When removing small pieces of sample from dough or gluten, the fine structure may be affected both by the cutting procedure and by the relaxation. Stretching of the pieces of underkneaded and developed dough and of gluten reduced the effect of relaxation by keeping the sample under tension approaching the conditions before cutting out the sample from the bulk. During the preparation the strong interfacial tension of clean water and other liquids may alter the protein structure, thus any contact to the air/liquid interface has to be carefully avoided during each step of the preparation. When preparing samples for the LM, many problems associated with TEM-preparation are avoided. Proteinaceous structures, however, are low in contrast and finer structures are difficult to resolve. Better results can be obtained by an electron microscope. However, the preparation of biological samples both for the SEM and TEM is more critical and frequently includes a freeze-drying step. Freezing of hydrated specimens is known to cause network-like artifactual structures in the presence of solubles or within hydrated material containing unbound water (Hermansson and Buchheim, 1981). These artifacts become apparent especially after freeze-drying but also after freeze-etching and freeze-fracturing. For an electron microscopist, with knowledge of the formation and of the appearence of such artifacts, it is essential either to reduce them or to consider them during evaluation (Moor, 1973; Plattner and Bachmann, 1982). This is of particular importance for this study as both the actual existing gluten structure and common freezing-artifacts exhibit network structures.

Therefore, the preparations were checked using CPD as an alternative to freeze-drying. As CPD is based on a completely different physical principle, its typical artifacts differ from that of freeze-drying (Boyde, 1978). Ideally, both methods should be used and the results compared to detect artifacts. If well adapted to the specimen, however, either method is suitable to reduce artifacts to practical invisibility. In our study, freeze-drying as well as CPD was used for preparing single hydrated flour particles, dough and gluten both for the SEM and the TEM and either method gave identical results at the magnifications applied. This indicates that the formation of visible artifacts has been prevented (Amend and Belitz, 1990b). Beside the EM methods mentioned, freeze-etching, negative staining, thin-sectioning and low-temperature-SEM were also used for this study (unpublished). The methods described, however, delivered the best results for our purpose. The EM results presented here are a representative selection out of about 4000 micrographs taken from more than 600 preparations (replicates not considered) at the TEM and the SEM.

### The Gluten Network

During kernel development, protein granules form and fuse within the endosperm cells forming a protein matrix extending between the starch granules (Bechtel and Barnett, 1986). Although the size of the granules is up to the µm-range (Bechtel et al., 1982), the hydrated gluten network observed in this work did not show any signs of being composed of globular bodies but appeared smooth in the light microscope and in the SEM. Also the fragments of finely ground, dry endosperm protein (wedge protein) did not show any globular structures of the

expected size (Amend and Belitz, 1990a, Fig. 1). Obviously the granules have fused perfectly to form a homogenous protein matrix, i.e. the gluten network.

**The Origin of Gluten**

From the experimental results the gluten protein is postulated to be preformed within each cell of the dry endosperm in the form of individual tiny networks. These networks exhibit viscoelastic properties on hydration. This is the conclusion from observations during spreading flour particles on a DTE solution, in which the strands first are extended elastically before they disrupt, due to disulphide cleavage by DTE. As the hydration in the presence of a reducing agent does not allow an oxidative polymerisation of the protein, it must exist preformed within the strands in a highly aggregated state. Therefore, the observed elasticity of the strands is due to water uptake only. The existence of gluten preformed in the kernel is also supported by the occurrence of viscoelastic gluten protein within the endosperm of premature wheat kernels that were investigated immediately after harvesting when still wet (Amend and Belitz, 1990b). These observations agree with the results of Dirndorfer et al. (1986), who found highly aggregated proteins in gluten that was isolated from flour under nitrogen atmosphere to prevent oxidation of protein components. The presence of oxygen, however, increased the amount of the high polymer protein fraction in a strong flour but not in a weak one.

**Dough Formation**

Based on our observations, dough forms from the tiny networks of three-dimensionally connected gluten protein strands that exist within each endosperm cell. Their elastic properties are immediately restored by the hydration. The formation of dough is basically a process of aggregation of these preexisting gluten networks by bringing the hydrated and sticky flour particles into close contact. The number of links between the protein networks of the individual flour particles increases during kneading. Cell wall residues isolating some of the networks from aggregating, are pulled apart. Sufficiently linked, the networks take up the kneading energy and are extended elastically. The increase in resistance towards extension that parallels the elongation of the strands, causes an increase in kneading resistance. Bidirectional extension occuring during kneading as a result of shear forces (MacRitchie, 1986) forms protein films both in dough and in washed gluten. Such a mechanism agrees remarkably well with the observations of Moss (1972). At the LM, he observed protein masses being stretched out into sheets during mixing. In section, the sheets appeared as long thin fibrils. Due to the method used, however, he could not clearly resolve the network structure of the protein whithin unextended and low extended flour particles which appeared as protein masses. The structural elements of gluten protein, strands and sheets have been observed also by other authors, using different methods (Khoo et al., 1975; Fretzdorff et al., 1982; Paredes-Lopez and Bushuk, 1983). Grosskreutz (1961), using both the TEM and small angle X-ray scattering, reported a sheet-like arrangement of gluten films within membranes. His preparation technique included freeze-drying of extended gluten membranes and was in parts quite similar to what we used. Despite the very low freezing rate of his samples, the results were similar to ours. This confirms the earlier findings (Amend and Belitz, 1990b) that extended gluten structures are rather resistant to freezing artifacts.

**Fine Structures of Gluten**

Observations at a macromolecular level of magnification revealed that during film formation by bidirectional extension of protein strands globular protein structures were transformed into platelet-like forms. The existence of globular gluten protein structures in a disordered close-packed arrangement is reported by Hermansson and Larsson (1986) based on freeze-etching studies of unextended gluten. Platelets have been proposed by Grosskreutz (1961) to be the basic structural element of gluten films. The thickness of the platelets of the highly extended films observed in our work by TEM (about 10 nm or less), agrees with the measurements of Grosskreutz by small angle X-ray scattering (6-9 nm). In contrast to the model of Grosskreutz, however, no free interstitial water was observed between the platelets. The platelets were found to form from coiled and twisted protein threads which are torn apart mechanically (Amend and Belitz, 1990c). In the literature, linear macromolecules frequently are postulated to exist in gluten and are attributed to glutenin (Huebner, 1977; Graveland, 1988; Ewart, 1989, and citations therein). Our observations indicate that both protein platelets and linear macromolecules exist in dough. Which of the two structures dominates depends on the kind of extension: bidirectional to form platelets, unidirectional to form linear forms. In either case, the basic structure in the relaxed state is a coiled up thread. Platelets transform into threads when treated with DTE or TEA. As the sheets still exist, the threads remain slightly elongated instead of coiling up and, therefore, are well observable. So far, the effects of TEA and DTE on the fine structure of gluten appear similar. The

coarse structure, visible in the light microscope, however, exhibits differences, when in contact with these substances (Amend and Belitz, 1990c), which require further investigations at the SEM level of magnification.

Protein sheets have been found to perforate at high tensions to form networks, the fibrils of which can become extremely thin (Figs. 17, 22; Amend and Belitz, 1990c). It is assumed, that these fibrils which are down to 3 nm in diameter, consist of highly extended single protein threads. As the threads usually are between 10 and 30 nm in diameter, extension probably has altered the tertiary structure of the molecules that build up the threads. Thus, the 3 nm fibrils may represent one or few peptide chains that have been pulled out from the molecules, the unextended parts of which remain as thickenings (Fig. 22).

The susceptibility of the tertiary structure of protein molecules towards mechanical stress is known from spreading proteins at the air/water interface (Donaldson et al, 1980). The thickness of the interfacial film formed by unfolded molecules is well below the diameter of the dissolved molecules as was demonstrated with bovine serum albumin (Amend and Belitz, 1990a). The threads in gluten sheets may undergo a deformation, similar to unfolding, when flattened down into platelets with a thickness below thread diameter.

The results presented demonstrate the usefulness of microscopy for investigating the process of breadmaking. Knowledge of the microstructure is a necessary precondition to understand dough rheology and these results cannot be obtained by methods other than microscopy.

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## Discussion with Reviewers

**R. Moss**: Could the authors indicate whether they have applied their microstructural approach to either a comparison of the pattern of dough development or a study of gluten microstructure in doughs made from wheats of different inherent strength ? Have the authors investigated the effect on gluten microstructure of some of the oxidizing agents used in breadmaking e.g., potassium bromate or azodicarbonamide ?
**Authors**: In this study, dough formation was investigated with one wheat variety without using additives. The effects of oxidizing agents on dough development have been studied by LM (Moss R (1974) Cereal Science 19: 557-561). Our intention was to understand dough formation in more detail before focusing on differences in variety or on the effect of additives.

**P.Resmini**: The authors should give more information about the freeze-drying treatment.
**Authors**: To maintain the good structural preservation obtained by freezing rates exceeding 10,000°C/s, samples were freeze-dried at -80°C overnight on the shrouded specimen table of a Balzers BAT 400 freeze-etch unit. These conditions are recommended for freeze-drying of biological specimens to avoid recrystallization (Steinbrecht RA and Müller M (1987) Freeze-substitution and freeze-drying in: Steinbrecht RA and Zierold K (eds.): Cryotechniques in biological electron microscopy. Springer Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo).

**P.Resmini**: Could the heating of the sample (85°C) modify the protein structure ?
**Authors**: The protein structure is, of course, strongly altered by this treatment. This changes, however, were not detected at the SEM (compare Fig. 11 in this paper with Fig. 7 in Amend and Belitz, 1990b). According to Hermansson and Larsson (1986, Food Microstruct. 5: 519-530), changes in the microstructure of the protein are not detectable in the TEM even after heating at 95°C.

**P.Resmini**: The diagram of Fig. 9 shows a remarkable increase of the length of protein strands (3 times and more) during kneading. Therefore the gluten seems to have especially viscous properties. Are the perforated structure of Fig. 9k and the protein network of Fig. 17 still elastic ?
**Authors**: We are presently developing a micromethod for the LM to measure quantitatively the rheological properties of single gluten strands. First results indicate that the strands exhibit especially elastic properties. When stretching a gluten membrane frequently by hand until the protein sheets are heavily perforated, their elastic properties decrease. So far this effect has not been measured quantitatively.

**P.Resmini**: The authors postulate that the gluten is already preformed in the dry kernel. Nevertheless I think that hydration causes important and impressive modifications in the native protein structure which give rise to a new protein complex with characteristic rheological properties. Therefore I don't agree with the authors about their hypothesis.
**Authors**: We agree that hydration causes important modifications in the protein conformation, rendering it cohesive, elastic and sticky. We believe, however, that this modifications in principle represent the reversion of the dehydration process at the end of maturity of the kernel, and that the arrangement of the matrix protein, filling the space between the starch granules, is conserved, independent of the state of hydration.

**P.Resmini**: The authors state that in the LM and SEM images the hydrated gluten network never exhibits globular bodies. On the contrary, freeze fracture images clearly show globular subunits (10 nm diameter) in the gluten matrix of pasta (Resmini P, Pagani MA (1983) Food Microstruct. 2, 1) and bread [Fretzdorff, B et al. (1982)]. Are these freeze- fracture features comparable to the loops and knots of protein threads (as could be surmised by their dimensions) ?

**Authors**: The globular bodies (10 nm diameter) can be resolved in the TEM only. Although we mainly focussed on freeze-drying and CPD for preparation, our freeze-etching studies on propane-jet frozen samples also exhibited irregular globular patterns comparable to the literature mentioned. These features may indeed represent unextended gluten protein structures.