Aspects of Sample Preparation for Freeze-Fracture/Freeze-Etch Studies of Proteins and Lipids in Food Systems. A Review

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Abstract

To select optimum specimen preparation methods and to correctly interpret freeze-fracture/freeze-etch micrographs of food systems a detailed knowledge of the individual steps of preparation -- i.e. chemical fixation of samples, their cryoprotective pretreatment, cryofixation, freeze-fracturing and -etching, and replication -- and of their influence on the appearance of different constituents, especially proteins and lipids, is necessary. Food systems show great variation in composition, structure and especially in their content of water -- e.g. molecular and colloidal solutions, oil-in-water and water-in-oil emulsions, gels suspensions, semi-solid systems such as cheese, dried systems such as milk powders -- thus requiring a careful variation of preparatory conditions.

Introduction

During the past two decades, the freeze-fracture/freeze-etch technique has become one of the major methods for preparing biological systems for electron microscopic studies. Considerable progress has been made in developing and modifying various preparatory techniques, in optimizing the individual steps of preparation, in improving the interpretation of micrographs and, last but not least, in recognizing the various types of artefacts which can develop during each of the individual steps of preparation. The present knowledge about the different aspects of freeze-fracture/freeze-etch sample preparation, primarily confined to biological systems, has been repeatedly compiled in the literature (3, 4, 9, 10, 20, 25, 28, 30, 33-36, 46, 53-61, 67).

Two characteristics of the freeze-fracture/freeze-etch technique are its applicability to systems with any given water content and the mainly physical nature of the individual steps of preparation by which the fine structure of proteins and lipids can be made visible down to molecular dimensions. Since proteins and lipids are the main constituents of many foods, and since their mutual interaction influences the physical properties of the products, the freeze-fracture/freeze-etch technique has become one of the major techniques used in studying the microstructure of foods.

Although the general progress in the freeze-fracture/freeze-etch methodology for biological specimens has influenced the preparatory methods for food systems and the interpretation of micrographs, many aspects of food sample preparation are different from those for normal biological systems. Some characteristic features of food systems are the finely dispersed state of proteins and lipids as e.g. in milk products, the extremely varying water content between different products, and the variability in the degree of aggregation of proteins and lipids as a consequence of processing treatments.
The main objective of this review is, therefore, to compile the various preparatory aspects and experience of freeze-fracture/freeze-etch studies of proteins and lipids in food systems. In individual sections, the different steps of sample preparation, i.e. chemical fixation, cryoprotective pretreatment, cryofixation procedures for aqueous and non-aqueous systems, freeze-fracturing, freeze-etching, and replication will be treated only so far as they are specifically related to the preparation of food samples or the visualization of their microstructure. For a more detailed information about the methodology and related problems the reader is referred to the general freeze-fracture/freeze-etch literature.

1. Chemical fixation

When the freeze-fracture/freeze-etch technique was introduced as an alternative to thin-sectioning, there was a general optimism that chemical treatments such as fixation with glutaraldehyde and osmium tetroxide would no longer be necessary, because the cryofixation of biological specimens, especially of tissue samples, was believed to preserve native structure. Although this is true in some cases, structural changes induced by the cryoprotective pretreatment (see Section 2) have been detected which could be largely eliminated by chemical prefixation with glutaraldehyde (9, 10, 25, 67).

Whether food samples need to be chemically fixed depends mainly on whether there is a risk that the cryoprotective agents introduce artefacts in unfixed samples. In any case, chemical fixation should only be performed under strictly controlled conditions in order to reduce possible structural side effects to a minimum.

In the following text a few personal experiences are summarized in order to draw attention to possible problems with chemical fixation. First of all it should be emphasized that the cross-linking effect of fixatives such as glutaraldehyde will induce more serious changes in sols than in systems with an already organized, i.e. cross-linked structure (gel). For example, the effect of glutaraldehyde on a protein solution might be the formation of artificial aggregates whereas the network structure of a system like Cottage cheese is probably further stabilized. We have observed such a distinct formation of aggregates in a 3.5% solution of β-casein (9). Without fixation a more or less uniform distribution of the individual protein particles (10-20 nm in diameter) was found (Fig. 1), whereas a 1-hour fixation with 1% glutaraldehyde produced a pronounced degree of particle aggregation (Fig. 2) which was accompanied by a visible increase in the turbidity of the solution.

Recently we observed another effect of glutaraldehyde which obviously has not been generally taken into account. We measured a considerable drop of pH value of about 0.4 units in skim milk fixed with 1% glutaraldehyde, although the fixing solution (10% glutaraldehyde in milk serum) had been adjusted to the original pH value of the milk. This pH drop resulted in an irreversible reduction of the viscosity of the milk of about 10%. This reduced viscosity has to be ascribed to structural changes of the protein particles. Since similar pH-related effects occurred only with caseins or whey proteins, it is obvious that a glutaraldehyde fixation of protein sols requires careful control of pH during the addition of the fixative and also, where possible, direct electron microscopical examination of possible fixation artefacts.

These few examples demonstrate that our present knowledge of the effects of chemical fixation, especially on molecular and colloidal solutions of proteins, is not sufficient to predict all the consequences of such a treatment.

Finally, experimental conditions will shortly be described which require chemical prefixation prior to any further preparatory steps. As will be further outlined in Sections 3, 4, and 6, the freeze-fracture/freeze-etch methodology enables us to make structural details visible in different

![Fig. 1 and 2. Freeze-fractured preparations of an unfixed (Fig. 1) and a glutaraldehyde-fixed (Fig. 2) 3.5% solution of β-casein, both cryoprotected with 33% glycerol. Aggregation of casein particles (A) occurs as a result of cross-linking by the fixative. Bars, 0.5 μm.](image-url)
ways by modifying the aqueous phase of the specimen. For all deep-etching experiments (see Section 6), we need pure distilled water (or at least a highly diluted aqueous phase) in order to make the true outer surface of the aggregates visible without risk of contamination from dissolved material. Since proteins are sensitive to such drastic environmental changes (e.g. casein micelles disintegrate into casein submicelles when the original milk serum is replaced by distilled water), their structural organization must be chemically fixed in advance. Furthermore, it will be shown that it can be advantageous to freeze-fracture and freeze-etch an originally aqueous system in a non-aqueous medium such as dioxane or ethanol (12, 27) in order to modify the fracturing characteristics or to improve the cryofixation. Of course, such experimental conditions require similar chemical pretreatments (glutaraldehyde and/or osmium tetroxide fixation as in preparing specimens for thin-sectioning) in order to stabilize the system during the dehydration step.

2. Cryoprotective pretreatment

The formation of ice crystals within highly hydrated specimens is caused by insufficiently high cooling rates during the so-called standard freezing, i.e., the immersion of the specimen into melting Freon, propane, nitrogen, etc. (see Section 3). Since these freezing artefacts modify the original structure they have to be overcome either by previous cryoprotection or by using one of the so-called ultrarapid freezing techniques (see Section 3). The general principle of cryoprotective pretreatment as a preparation step in the freeze-fracture/freeze-etch technique has been repeatedly reviewed (25, 34, 61) and, therefore, further information can be confined to special problems related to the preparation of foods for electron microscopy.

Whether a food sample needs any cryoprotection at all must be decided according to the occurrence and/or degree of structural modification. If only certain structural components or phases of a system are of special interest, and if they are not affected by ice crystallization, these artefacts may be tolerated.

The commonly preferred cryoprotectant is glycerol. If a homogeneous mixture of 2 volumes of distilled water and 1 volume of glycerol is frozen by the standard freezing method and properly handled during the entire freeze-fracture/freeze-etch procedure (in order to avoid any recrystallization), the degree of ice crystallization is tolerable.

We, therefore, dilute aqueous liquid food systems, such as colloidal solutions, emulsions, or suspensions to 33% glycerol so that the largely artefact-free freezing of the aqueous phase is guaranteed. The homogeneous mixing of a liquid sample with glycerol is generally achieved by careful magnetic stirring for about 5 min. If an aqueous food sample has a non-liquid, i.e., gel-like structure, small pieces of this specimen are immersed in adequately buffered solutions with the above-mentioned glycerol concentration. After 10 to 20 min, pieces of 1 to 2 mm are usually completely penetrated.

Whether the standard freezing procedure produces detectable ice crystals in a specimen and, therefore, requires cryoprotection, depends not only upon the absolute content of water but also upon whether the water is mainly free or is more or less bound. Therefore, many cheese varieties such as Gouda, Camembert, or process cheese, having a water content of up to 60 or 70% within the fat-free substance, can be prepared without any cryoprotection (29, 62), although pretreatments of such samples in a 30% solution of glycerol also have been described (21).

According to our experience, a glycerol concentration of 20% does not suffice to prevent significant ice crystallization in a system like skim milk, although such conditions have been used (24). The corresponding micrographs, therefore, do not appear to be free of artefacts.

The question, which may arise with reference to the cryoprotection step, is to what extent it may introduce structural changes, i.e., artefacts, within the specimen. With glycerol such effects have been observed within biological systems, e.g., swelling of mitochondria, transformation of laminar endoplasmic reticulum into a vesicular state, or plasmolysis within plant cells (9, 10, 34, 67).

Not too much is known about the exact influence of glycerol on food samples. In an earlier study we compared the size distribution of casein micelles in a glycerol-free skim milk preparation with glycerol-pretreated preparations and varied the glycerol concentration between 20 and 60% (v/v). There were no significant differences between the different samples (49). Of course, this result does not exclude possible negative effects on other protein systems. Possibly, the monomer-polymer equilibrium in protein solutions is disturbed in the presence of higher glycerol concentrations, but such studies have not yet been made. In another case we observed considerable swelling of a heat-induced 12% soy protein gel when the sample was immersed into 30% glycerol (23).

These few observations make it clear that the glycerol treatment has to be carried out with some caution although glycerol impregnation is still an almost ideal method of cryoprotection.

Finally, it should be mentioned that glycerol is very suitable as a binding medium between a specimen of higher density (e.g., hard cheese or systems like butter or margarine) and the specimen holder (29, 43-45, 62). In addition to the benefit of higher mechanical stability of the specimen during freeze-fracturing, the removal of the specimen from the holder after thawing is facilitated using this water-soluble intermediate layer.

3. Cryofixation of aqueous systems

A complete physical fixation of the original fine structure of a specimen by quick freezing is the prerequisite for the optimum use of the freeze-fracture/freeze-etch technique. The main problem encountered during freezing is the formation of ice crystals, which causes further alteration of the original structure by concentrating dissolved or particulate constituents in the 'eutectic' phase between the ice crystals. The crystal size is mainly a function of the freezing rate. An ideal degree of cryofixation is achieved if the crystal size
measures approximately 10 nm. For highly hydrated biological specimens and food samples with a similarly high water content, cooling rates of 10,000 K per second and more would be necessary to achieve this crystal size (34). Nowadays, two different principles of freezing are distinguished. The so-called standard freezing technique involves the direct immersion of the specimen (1 to 2 mm³ in volume) in coolants such as melting Freon 12 (dichlorodifluoromethane) or Freon 22 (monochlorodifluoromethane) at 113 K, liquid propane at 83 K, or melting nitrogen at 63 K (25, 34, 61). This more popular procedure results in cooling rates of only some 100 K/sec and, therefore, generally produces ice crystal artefacts in highly hydrated samples which can only be suppressed by adequate cryoprotection (see Section 2). Alternatively, several so-called ultrarapid freezing techniques have been developed which result in considerably higher cooling rates (2, 4, 11, 13, 17, 19, 37, 47). Although a cryoprotective pretreatment can be omitted when using these techniques, the high degree of structural preservation is restricted to very small sample volumes of about 10⁻⁶ mm³ or very thin samples (10 - 50 μm thickness). The capabilities and limitations of the two principles of freezing are described in the following sections.

3.1. The standard freezing technique

Although the standard freezing technique is an effective and straightforward procedure, the amount of ice crystal damage to be expected within a sample and the need for a cryoprotective pretreatment must first be determined. The easiest way to check this is to cryofix such a sample with and without cryoprotection, and, if possible, also to compare the resulting fine structure with that obtained by other preparatory techniques, e.g. thin-sectioning. Ice crystal formation during freezing depends not only upon the absolute water content but also on the degree of its orderliness within the sample, as has been outlined in Section 2. As a general rule, structural damage by ice crystal formation has to be expected in systems which contain more than 80% water.

Ice crystals in such highly hydrated systems normally measure approximately 1 μm. This is demonstrated in Fig. 3, showing a skim milk sample, and in Fig. 4, showing a 12% heat-coagulated soy protein gel (17, 23), both frozen by direct immersion into melting Freon without any cryoprotection. These two examples demonstrate the main features of inadequate freezing. The ice crystals appear as very smooth areas in purely freeze-fractured, i.e. unetched, preparations which are surrounded by the 'eutectic' phase, whereas the dissolved constituents (salts, lactose etc.) and the particulate constituents (small lipid particles, casein micelles, soy protein particles) are concentrated as thin lamellae. If the ice phase had been removed, the 'eutectic' phase would appear raised and would present a three-dimensional honeycomb-like network.

Figures 3 and 4 demonstrate further that occasionally some particulate constituents may remain within the ice phase. A comparison of the average ice crystal size in both samples measures approximately 10 nm. For highly hydrated biological specimens and food samples with a similarly high water content, cooling rates of 10,000 K per second and more would be necessary to achieve this crystal size (34). Nowadays, two different principles of freezing are distinguished. The so-called standard freezing technique involves the direct immersion of the specimen (1 to 2 mm³ in volume) in coolants such as melting Freon 12 (dichlorodifluoromethane) or Freon 22 (monochlorodifluoromethane) at 113 K, liquid propane at 83 K, or melting nitrogen at 63 K (25, 34, 61). This more popular procedure results in cooling rates of only some 100 K/sec and, therefore, generally produces ice crystal artefacts in highly hydrated samples which can only be suppressed by adequate cryoprotection (see Section 2). Alternatively, several so-called ultrarapid freezing techniques have been developed which result in considerably higher cooling rates (2, 4, 11, 13, 17, 19, 37, 47). Although a cryoprotective pretreatment can be omitted when using these techniques, the high degree of structural preservation is restricted to very small sample volumes of about 10⁻⁶ mm³ or very thin samples (10 - 50 μm thickness). The capabilities and limitations of the two principles of freezing are described in the following sections.

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Ice crystals in such highly hydrated systems normally measure approximately 1 μm. This is demonstrated in Fig. 3, showing a skim milk sample, and in Fig. 4, showing a 12% heat-coagulated soy protein gel (17, 23), both frozen by direct immersion into melting Freon without any cryoprotection. These two examples demonstrate the main features of inadequate freezing. The ice crystals appear as very smooth areas in purely freeze-fractured, i.e. unetched, preparations which are surrounded by the 'eutectic' phase, whereas the dissolved constituents (salts, lactose etc.) and the particulate constituents (small lipid particles, casein micelles, soy protein particles) are concentrated as thin lamellae. If the ice phase had been removed, the 'eutectic' phase would appear raised and would present a three-dimensional honeycomb-like network.

Figures 3 and 4 demonstrate further that occasionally some particulate constituents may remain within the ice phase. A comparison of the average ice crystal size in both samples

Fig. 3. (Facing page). A skim milk sample, cryofixed by the standard freezing technique without previous cryoprotection, segregates into ice crystals (IC) and a network-like eutectic phase (EP). This phase contains solutes (salts, lactose) and particulate constituents (casein (C) and whey proteins). Note that some casein micelles (CM) and a small lipid vesicle (L) remained within the ice phase. Bar, 0.5 μm.

Fig. 4. An uncryoprotected heat-coagulated 12% soy protein gel exhibits a pattern similar (although with smaller ice crystals) to the skim milk (see Fig. 3). This is probably due to the higher protein content and the gel structure itself. This artificial network is considerably coarser than the true gel network which is shown in Fig. 9-11. This preparation has been slightly freeze-etched. (IC = eutectic phase; P = isolated protein particles within ice crystals). Bar, 0.5 μm.

Fig. 5 and 6. Microscopically small water (serum) droplets in water-in-oil dispersions like margarine (Fig. 5) and butter (Fig. 6) solidify without significant ice crystal growth during the standard freezing technique. In a freeze-fractured preparation (Fig. 5), protein aggregates (P) are clearly detectable in such droplets, whereas freeze-etching (Fig. 6) produces a coarse pattern. The fine structure of the fat phase indicates crystallized (C) as well as originally liquid (L) components. Bars, 0.5 μm.

Fig. 7 and 8. Casein micelles (C) are partially adsorbed at the surface of the droplets formed when skim milk (SM) is either prepared by spray-freezing (Fig. 7) or by the emulsion freezing technique (Fig. 8). BB = butyl benzene phase; PA = paraffin phase. Bars, 0.5 μm.

Figures 7 and 8. Casein micelles (C) are partially adsorbed at the surface of the droplets formed when skim milk (SM) is either prepared by spray-freezing (Fig. 7) or by the emulsion freezing technique (Fig. 8). BB = butyl benzene phase; PA = paraffin phase. Bars, 0.5 μm.

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Figures 3, 4, 5, 6, 7, 8 show various micrographs of freeze-fractured food samples.

- Figure 3: Images with labels IC, CM, L, EP
- Figure 4: Images with labels IC, EP
- Figure 5: Images with labels C, P
- Figure 6: Images of a dense, circular structure
- Figure 7: Images with labels BB, C, SM
- Figure 8: Images with labels PA, C, SM

Each figure may contain additional annotations or labels not visible in the provided images.
Comparable structures have been found in gelatin gels (7, 64), gels of α- and β-casein (8), vicia faba protein gels (48), and potato starch gels (63). In all these studies the authors have considered the resulting network structure as the real gel structure instead of representing freezing artefacts.

In contrast, Tomka and Spühler (65) have carefully taken into account the consequences of inadequate cooling rates during freeze-etch studies on gelatin gels. They realized that the conditions during standard freezing produce network structures which are far too coarse to be considered to be the true original structures. They demonstrated, however, that if only small gel droplets of 0.3 mm in diameter were cryofixed by immersion into melting Freon, the ice crystallization diminished towards the surface of the droplet and the true gel structure became visible in a narrow zone. The characteristics and dimensions of the gelatin network were found to be similar to those observed with chemically fixed and stained thin sections.

These results demonstrate very clearly that gel structures should be cryofixed under very controlled conditions in order to avoid misleading conclusions. The application of ultrarapid freezing techniques, therefore, appears to be the most appropriate for such systems (see Section 3.2).

Whereas the formation of freeze artefacts is highly probable in systems with approximately 80 and more per cent of 'free' water, not too much is known about the freezing properties of systems with lower contents of 'free' water. As already mentioned in Section 2, the standard freezing of systems like Gouda, Camembert, and process cheese, which still have 60-70% water within the fat-free substance, does not produce ice crystals of measurable size (29, 62). But in these systems, the water is uniformly distributed in a very dense matrix of proteins and peptides and, therefore, has to be considered as mainly 'bound' water.

Systematic studies on the freezing properties of systems having a tower water content but a continuous water phase, e.g. oil-in-water dispersions (emulsions), have not yet been made. In contrast, dispersions (emulsions) of the water-in-oil type such as margarine and butter have been studied by freeze-fracturing/freeze-etching (14, 45). The finely dispersed aqueous phase with droplet diameters of up to 10 μm and more exhibited a very low degree of ice crystal formation as can be seen in Fig. 6 (margarine, freeze-fractured) and in Fig. 6 (butter, freeze-etched). The reason for the absence of larger ice crystals in these droplets cannot be due to a higher cooling rate because of the low thermal conductivity of the fat phase. Perhaps the greater degree of supercooling of such small volumes is responsible for this effect.

Starting from this observation, we developed a special freezing technique, the so-called oil emulsion freezing technique, which will be discussed in greater detail in Section 3.2. However, the results obtained with margarine and butter, the water content of which is approximately 15% (v/v), do not allow further conclusions to be made for such systems with higher water contents.

3.2. Ultrarapid freezing techniques

Great efforts have been made to overcome the limitations imposed by the insufficiently high cooling rates of the standard freezing techniques. The aim was not only to avoid any cryoprotective pre-treatment and chemical fixation but also to fix the original fine structure just at the moment of freezing. This seems important especially for highly dynamic systems, and to allow a real freeze-etching, i.e. sublimation of ice to any desired depth for making surface structures visible.

However, it has been recognized that a negligible growth of ice crystals during the freezing of pure water or highly hydrated systems can only be achieved if the sample volume or thickness is substantially smaller than during standard freezing, namely of a magnitude of 10 μm (diameter of droplet-shaped specimens or thickness of thin-layer specimens). This limitation prevents a general application of ultrarapid freezing techniques and, in addition, requires highly sophisticated equipment. Nevertheless, considerable progress has been made by applying such freezing techniques to biological systems as well as to food systems.

A very effective ultrarapid freezing technique is the spray-freezing technique of Bachmann and Schmitt (2-4). It is only applicable to fluid systems such as colloidal solutions, emulsions, and suspensions, because the sample has to be sprayed directly into liquid propane (83 K), thereby forming very small droplets (up to 10 μm and more in diameter). In addition to a possible supercooling, the temperature of such small droplets is reduced so rapidly that under proper experimental conditions the size of ice crystals does not exceed values of 5-15 nm, which in most cases has to be considered as a sufficient degree of cryofixation.

The normal method of handling the frozen droplets is to replace the propane by another suitable liquid, e.g. n-butylbenzene (melting point 185 K), at temperatures well below the recrystallization temperature (e.g. 188 K) and finally to freeze 1-2 mm² of a highly concentrated suspension of the frozen droplets in this second liquid mounted on normal freeze-fracture specimen holders by immersion into liquid nitrogen.

This technique has been often used to study the size and shape of hydrated macromolecules such as ferritin (3), fibribogen (5), and enzyme complexes such as pyruvate dehydrogenase (26). The main advantage of this preparatory approach is that the dissolved macromolecules can be made visible by subliming the surrounding ice to any desired depth, if the concentration of salts etc. is low enough. If two different macromolecular solutions are independently sprayed into the same volume of propane and one solution is labelled with known particles such as polystyrene latex spheres, molecular weight (particle weight) determinations can be performed simply by counting particles per unit area on the micrographs (3).

We have used this technique for studying the monomer-polymer equilibrium of β-casein (16) and the size and molecular weight of α-lactalbumin and β-lactoglobulin (51).
Should this technique be applied to systems containing aggregates which react sensitively to shear forces, attention should be paid to possible structural changes (deformations) during droplet formation. In protein-containing systems such as skim milk we observed an adsorption of casein micelles at the droplet surface. This must be taken into account, especially for quantitative measurements (Fig. 1, 2).

Recently further ultrapid freezing techniques have been developed which are also mainly applicable to fluid systems or systems which allow for preparation as thin films. All these alternative methods have not yet been applied to food systems but appear to be equally suitable for liquid samples and possibly also for gel-like samples. Lepault and Dubochet (30) demonstrated that particulate suspensions, when put between two 25 μm-thick copper pedestals spaced by a 0.03 mm thick microscope grid (sandwich technique) could be cryofixed with substantial ice crystal formation by plunging into nitrogen slush. This type of specimen has to be freeze-fractured in a special apparatus (31). The so-called propane jet freezing technique represents another alternative method for ultrapid freezing of aqueous specimens. In a special apparatus, two jets of liquid propane are blown simultaneously from opposite directions onto a thin film (approximately 12 μm), sandwiched between conventional copper grids (32) and supported in a reduced ice crystal growth due to a high cooling rate. Recently, a special application of this technique has been described for freezing samples from temperatures higher than room temperature and thereby studying phase transitions of phospholipids (66). Robards and Severs (47) recently compared the cooling rates achieved within such a sandwich specimen when frozen in a propane jet device and when alternatively plunged by hand into liquid propane, and found no substantial differences.

As another alternative, the so-called oil-emulsion freezing technique was described here, although resulting low degree of ice crystallization is rather induced by greater supercooling than by faster cooling rates. As already described in Section 3.1., microscopically small water (serum) droplets in systems like butter and margarine exhibit a high degree of cryofixation despite comparably low cooling rates (14, 45). Similar effects are obtained if any hydrated system whatever is finely dispersed in an oil phase and this emulsion is frozen in quantities of 1-2 mm³ on normal specimen support by immersion into melting Freon or other suitable coolants (12, 13, 17, 23). This particular freezing technique does not require any sophisticated equipment and need not be carried out under stringent experimental conditions. For the oil phase we generally use paraffin (type: viscous), if necessary, with some oil-soluble emulsifier (monoglycerides or polyglycerol esters). One part of the aqueous phase is dispersed mechanically into 10-20 parts of the oil phase so that the majority of the droplets have a diameter between 5 and 10 μm and the droplets are homogeneously distributed within the oil phase. Further details will be found in the literature (12, 13, 17, 23).

We applied this technique repeatedly to fluid systems like milk, to systems with an organized structure such as animal tissue, and also to systems which are able to undergo (reversible or irreversible) sol-gel transformations. Of course, the mechanical disintegration step can introduce structural damage but this effect has to be evaluated individually. For example, casein micelles in skim milk or protein particles in general show a tendency to be accumulated at the oil-water interface in a manner similar to that seen with spray freezing (Fig. 8). Such uneven distributions have to be taken into account for quantitative measurements (e.g. particle densities).

A special application of this freezing technique is the study of sol-gel transformations (16, 23). For this purpose a gelling system is dispersed within the oil in the sol state, the individual sol droplets are protected against fusion by an effective emulsifier and then the emulsion is kept under such conditions (heating or cooling) that gelation occurs within the small droplets. The structure of the gelling phase can be cryofixed at any desired state. We have demonstrated the effectiveness of this procedure with heat-coagulated soy protein gels (16, 23). Fig. 9 shows the distribution of the protein particles within this type of gel after freeze-fracturing (no etching!) and Fig. 10 and 11 show this gel after freeze-etching. Compared with the coarse artificial network obtained after standard freezing of gel pieces (Fig. 4), it is obvious that the true structure is characterized by a much denser and finer network.

Recently we applied this technique to studying sol-gel transformations of gelatin. Fig. 12 shows the structure which is obtained when a 1% sol of gelatin (dispersed in paraffin) is frozen from a temperature of 333 K, whereas Fig. 13 shows the structural transformations which occurred within the small droplets when the emulsion was stored for 2 days at 277 K and was then frozen without rewarming. Both micrographs represent conditions after the sublimation of ice, i.e. after freeze-etching. Although the fine network of the gel state, which largely conforms to the characteristics reported by Tomka and Spühler (65), can be seen very clearly, the micrograph of the gelatin sol also shows a certain, though considerably lower degree of aggregation. Whether this somewhat uneven distribution of the particles reflects the true original structure or whether a slight uncontrolled reduction in the sample temperature during handling caused this aggregation, could not yet be ascertained.

4. Cryofixation of non-aqueous media

For some liquid substances, which are either components of foods such as oils and fats or are used as embedding media, e.g. glycerol, paraffin, and polyethylene glycol, the cooling rates, which are obtained during the conditions of standard freezing, suffice to suppress any recognizable crystallization. We have used pure glycerol, paraffin (type: viscous), and polyethylene glycol (mol. weight 400) for suspending different kinds of dried powdered milk products and thereby achieved suitable conditions for freeze-fracturing such systems (18). The structural characterization of semi-solid fat
Fig. 9, 10, and 11. Freeze-fractured (Fig. 9) and freeze-etched (Fig. 10 and 11) samples of a 12% heat-coagulated soy protein gel prepared for electron microscopy using the oil emulsion freezing technique (i.e. dispersion of the sol in paraffin and thereafter heating the entire system to 358 K for 30 min for sol-gel transformation). The protein particle density in Fig. 9 characterizes the type of network of this gel. Locally, parts of single protein strands (PS) become visible. After freeze-etching (175 K, 1 min), at a higher magnification (Fig. 11) the arrangement of protein (P) and protein-free spaces (PF) reveals the true dimensions of this network. Bars in Fig. 9 and 10, 0.5 \( \mu \)m; bar in Fig. 11, 0.1 \( \mu \)m.

Fig. 12 and 13. For legends see the facing page.
Using the oil emulsion freezing technique, reversible structural changes between the sol state (Fig. 12) and the gel state (Fig. 13) of a 1% gelatin-in-water system can be studied simply by adequate tempering of the emulsion (at 333 K for the sol state and at 277 K for the gel state) before cryofixation. Both figures show freeze-etched preparations (163 K, 5 min). Whereas the gelatin gel has a pronounced fibrous network (Fig. 13), the protein particles seem to be loosely aggregated in the sol state (Fig. 12). (Possibly this slight aggregation does not reflect the true situation at 333 K, but is a result of an uncontrolled drop in the temperature during handling of the specimens before freezing). Bars, 0.5 μm.

Fig. 14. Casein micelles (CM) in skim milk (33% glycerol) occasionally show strong plastic deformations (PD) after freeze-fracturing. The smooth structure of the milk serum phase indicates the absence of any contamination or sublimation artefacts. Bar, 0.5 μm.

Fig. 15, 16, and 17. Rennet-coagulated casein micelles (fixed with glutaraldehyde) are used as an example to demonstrate the striking differences which occur during freezing and fracturing in aqueous and non-aqueous media. In Fig. 15, the original coagulum (cryoprotected with 33% glycerol) has been freeze-fractured. In Fig. 16 (freeze-fractured) and Fig. 17 (freeze-etched), the coagulum has been dehydrated to 100% dioxane. In an aqueous medium, the casein aggregates (C) are always cross-fractured, whereas in a non-aqueous medium such as dioxane these aggregates are predominantly surface-fractured (Fig. 16). This surface-fracturing either results in views of the particle surface (1) or of the complementary depressions in the dioxane matrix (2). To sublime the dioxane matrix (Fig. 17), freeze-etching was performed for 2.5 min at 173 K; the specimen was thereafter cooled to 143 K for replication. Bars, 0.5 μm.
phases is also enhanced because of these favourable freezing characteristics. The distribution of liquid and crystallized fat in systems such as butter and margarine can be clearly determined in micrographs due to the amorphous appearance of the originally liquid fat (14, 43-45). Similar distinctions between the liquid and crystallized states of fat fractions (before cryofixation) can be made within freeze-fractured fat droplets, i.e. if the fat phase is present in a dispersed state such as in a dairy cream (15).

Furthermore, organic solvents such as toluene, dioxane, and ethanol have been substituted for the water of originally aqueous systems (coagulated casein micelles, yoghurt, soy protein gels, and also animal tissues). Such solvents were used with chemically fixed systems (12, 23, 27). During cryofixation of such specimens under standard conditions, the extent of crystallization of the solvents appeared to be much less pronounced than with water. This special modification of freeze-fracturing/freeze-etching previously aqueous systems could, therefore, be advantageous not only because of improved structural preservation during cryofixation, but also because of different fracture characteristics (see Section 6) and because of the possibility of deep etching (see Section 7).

5. Freeze-fracturing

The freeze-fracturing of a cryofixed specimen can be performed in different ways and at different temperatures. The optimum conditions depend largely on the type of specimen, on the type of equipment, and on whether a controlled low-temperature sublimation ('freeze-etching') of volatile constituents such as water shall follow the cleavage of the specimen. For further details, the reader is referred to the literature (25, 55, 59, 61).

For producing a freeze-fractured preparation, it is necessary to replicate the freshly cleaved surface of the specimen immediately after fracture and to avoid any contamination or sublimation which may introduce peculiar artefacts (46). In our laboratory, where a Balzers BA 360 M freeze-etching unit is used, usually a specimen temperature of 153 K is chosen for performing the freeze-fracturing, and the evaporation unit (electron gun) is started shortly before the final fracture. Under such conditions, highly reproducible freeze-fractured preparations without any recognizable contamination or sublimation artefacts are obtained.

Proteins and lipids are affected by the cleavage process in a distinctly different way. If we freeze-fracture single protein molecules or small oligomers in solution, those particles are mostly plasticly deformed or even torn apart. After replication they become visible as small, mostly 10-20 nm wide particles, always protruding out of the surrounding ice (50). The apparent size of these deformed particles is related to the actual (molecular) weight and also to their structure, i.e. molecules with a loose, random coil conformation (e.g. 8-casein) generally appear larger in freeze-fractured replicas than compact globular proteins (e.g. 8-lactalbumin, lactoglobulin). As an example for this typical fracturing of molecular protein aggregates, see the 8-casein particles in Fig. 1 and 2 or the soy protein particles in Fig. 9.

If proteins form larger aggregates, e.g. casein micelles in milk or even larger aggregates due to various clotting processes, the fracture plane always runs through such highly hydrated aggregates at the same level as through the surrounding ice but the molecular particles within such aggregates are affected similarly to isolated particles.

The plastic deformation during freeze-fracturing does not only occur with macromolecules in solution but also in biological systems, especially within biomembranes and crystals (e.g. catalase), and in various types of non-biological polymers (20, 30, 54, 55). Although the degree of deformation is reduced with decreasing fracturing temperature, it may still occur at temperatures of 4 K (54). This phenomenon is probably related to a dissipation of heat as a result of the fracturing event. However, it should be taken into account that at higher cleavage temperatures elastic recontraction or collapse of deformed structures, either immediately after the separation of the opposing fracture faces or during etching and replication, may lead to replicas showing a considerably higher degree of complementarity (54). We have found that occasionally complete casein micelles are greatly deformed during freeze-fracturing (at 153 K). This is demonstrated in Fig. 14. The horn-like protrusions resemble those of latex spheres (54). In spite of the effects of plastic deformation of proteins, freeze-fractured preparations (without any contamination or sublimation artefacts) of colloidal protein systems are generally most informative and also especially suited for quantitative measurements (32, 49).

It is an additional experience that the structural characterization of very dense protein matrices such as in cheese is facilitated by studying only freeze-fractured preparations (29, 62). These matrices differ from normal protein aggregates by the gradual enzymic degradation of protein molecules into peptides. In freeze-fractured preparations the relatively small differences between size, shape, and density of the remaining particles are more evident than after a subsequent sublimation of ice (compare Fig. 33 and 34).

If proteins are studied in the dry state, as e.g. in dried milk or in dried protein concentrates, the fracturing characteristics and the fine structure resemble those of the hydrated state (18). As a result of our structural comparison between different dried milk products we found distinct differences in the apparent size of globular whey protein particles and randomly coiled casein particles.

The freeze-fracturing characteristics of protein aggregates in organic solvents such as toluene and dioxane differ markedly from those in aqueous media (12), as most aggregates are no longer cross-fractured but surface-fractured. This fundamental difference is demonstrated in Fig. 15, 16, and 17, presenting micrographs of the same type of sample (rennet-coagulated casein micelles fixed with glutaraldehyde). Recently Kalab (27) freeze-fractured a (chemically fixed) yoghurt sample in ethanol and subsequently dried it for scanning electron microscopic studies. He observed similar differences between the fracturing of the protein aggregates in an aqueous and in the
Fig. 18, 19, 20, and 21. Fat globules in fresh, not cooled milk exhibit 4 major fracture types. (I): cross-fractured globules, in which the fat phase (F) is exposed. Occasionally layered structures (L) appear, which are still different from really crystallized fat. The fat globule membrane (M) is exposed only locally (Fig. 18). (II): exposure of the outermost layer of the fat core due to fractures between the inner surface of the fat globule membrane and the fat core. Fig. 19 shows the smooth appearance of this surface which is only interrupted in a limited area (1), where the fracture went deeper into the fat phase. (III): The complementary view after such a fracture reveals the inner surface of the fat globule membrane (iM) to which small portions of fat (F) occasionally adhere (Fig. 20). (IV): Only rarely do fractures between the outer surface of the fat globule membrane (oM) and the serum occur. In Fig. 21 the fat globule membrane has been partially removed, exposing the outermost layer of the fat core (F). In Fig. 21 ice crystallization occurred despite glycerol cryoprotection. This is probably due to inadequate mixing with the milk. (IC: ice crystals; EP: eutectic phase). Bars, 0.5 μm.
The freeze-fracturing characteristics of lipid phases and lipid aggregates are determined mainly by the amounts and spatial distribution of molecularly well-ordered ('liquid crystalline' and crystalline) and non-ordered (liquid) lipids, and also by the size of aggregates and the type of interfacial layers. Milk and dairy products are well suited to demonstrate the main differences in appearance. On the one hand, the milk fat is a complex mixture of different high-melting fat fractions which exhibit peculiar crystallization and melting phenomena, and on the other hand, the state of dispersion of the fat phase and the type of interfacial layers undergo severe changes during processing. Within the lactating cells of the mammary gland, fat droplets (several micrometers in diameter) are formed. During secretion they are enveloped in (apical) plasma membrane, the so-called milk fat globule membrane (MFGM). This interfacial layer is responsible for the high emulsion stability of the fat phase in fresh milk (39).

Although the milk fat contains fat fractions with melting points higher than body temperature, the fat is totally liquid when secreted and crystallization processes do not start until fresh milk is cooled below 293 K. Freeze-fracturing of fat globules in fresh uncooled milk results in the following fracture characteristics: They are either cross-fractured at the same level as the surrounding aqueous phase or are fractured along their periphery. Cross-fractured fat globules (Fig. 18) exhibit the typical fine structure of non-crystallized fat. Although it largely appears amorphous, smaller areas with a very characteristic layered structure can frequently be found. According to our experience, such layers seem to represent a (non-crystallized) state where triglyceride molecules of similar length are somewhat loosely packed together in monolayers (15). These layered structures occur predominantly in a temperature range between body temperature and the temperatures where true crystallization starts within the fat globules (230-280K). There are no indications so far that these structures are formed during cryofixation. Cross-fractured fat globules only occasionally reveal small portions of the MFGM (see Fig. 18). Cross-fractured areas of the MFGM very often show numerous small (approx. 10 nm wide) particles which should largely represent membrane proteins (enzymes, glycoproteins etc.) because up to 60% of the dry weight of the MFGM is protein (39). Peripheral fractures of fat globules in (uncooled!) fresh milk occur predominantly at the inner surface of the MFGM, i.e. between the MFGM and the fat core, and only very rarely at the outer surface, i.e. between the MFGM and the milk serum. An internal cleavage of the MFGM as in biomembranes (55, 57, 61) almost never occurs in expressed milk. A bilayer structure has only occasionally been found in a narrow zone in cross-fractured MFGMs (6).

To demonstrate the three possible cases of surface fracture of fat globules in fresh milk, compare Fig. 19, 20, and 21. Fig. 19 shows the smooth outer surface of the fat core, interrupted in a limited area to reveal underlying monolayers of fat. The complementary event is shown in Fig. 20, where the inner surface of the MFGM, with fat adhering to a limited area, is exposed. A view of the true outer surface of the MFGM is seen in Fig. 21. It can be seen that certain parts of this MFGM must have been removed with the complementary part of this fracture.

An explanation for the preferential fracturing between the inner surface of the MFGM and the (previously) liquid fat core could be a much stronger interaction of the phospholipid molecules (and membrane proteins?) with the milk serum than with the fat. The reason for the absence of internal fractures of the MFGM which one would expect if the original phospholipid bilayer structure of the plasma membrane still existed, is not yet fully understood. Possibly a reorganization of membrane constituents after secretion takes place due to the presence of (apolar) triglycerides at the inner surface of the MFGM. This could, of course, substantially alter the fracturing characteristics. Alternatively, freeze-fracture studies on the secretion mechanism of fat globules indicate a 'clearing of membrane particles' on those parts of the plasma membrane which are directly involved in the secretory event (40).

During homogenization of fresh milk, the fat globules are strongly reduced in size, their total surface area increases correspondingly (often ten-fold and more) and a new type of interfacial layer is formed, consisting mainly of adsorbed milk proteins, predominantly caseins. When homogenized milk (Fig. 22) or milk products (Fig. 23) are cryofixed from temperatures at which the milk fat is completely liquid, the fracturing characteristics somewhat resemble those described for fresh milk. Cross-fracture through fat globules largely prevail, making the newly adsorbed protein layer clearly visible (Fig. 22). Only rarely do surface fractures occur. These exhibit a very smooth structure. Since adsorbed protein particles would be easily detected, the observed smoothness demonstrates that the fracture occurs again between the outer surface of the fat core and the inner surface of the 'secondary' fat globule membrane.

As soon as unhomogenized or homogenized milk and milk products are cooled and the milk fat begins to crystallize, the freeze-fracturing characteristics undergo substantial changes. Fat crystals are easily cleaved between individual monolayers and are easily distinguished from liquid fat. The spatial arrangement and shape of crystallized fat within a globule depend largely upon the cooling conditions (temperature and time). This is especially evident during the 'physical ripening' of cream (15). A common feature of most crystallized milk fat globules is the growth of peripheral monomolecular crystal layers directly below the original MFGM or the 'secondary' (protein) membrane.

A main consequence of this peripheral crystallization process is a predominant cleavage within these largely concentric monolayers. As an example, a dairy spread with a homogenized fat phase (average fat globule diameter similar to the coffee cream shown in Fig. 23) is shown in Fig. 24. Neither cross fractures nor fractures along the outermost layer of the fat core, i.e. between the fat and the interfacial protein layer, seem to exist. Fig. 25 and 26 show how unhomogenized fat globules have been freeze-fractured in a homogenized cream (Please note that Fig. 25 has only about 1/3 of the magnification of Fig. 24). These micrographs...
Fig. 22. Uncooled homogenized milk fat globules are mostly cross-fractured (1) but also show fractures along the inner surface of the fat globule membrane which consists mainly of absorbed casein particles (C) or micelles. (2: view of the fat core; 3: partial view of the inner surface of the fat globule membrane). Bar, 0.5 μm.

Fig. 23. The prevailing occurrence of cross-fractures through fat globules which are liquid before cryofixation is striking in this fractured preparation of a coffee cream (10% fat, homogenized). Peripheral fractures at the fat-membrane interface (M) appear only rarely. Bar, 1 μm.

demonstrate that peripheral fractures within the layers of crystallized fat still prevail but, in addition, cross-fractured fat globules can be detected frequently. Again, fractures between the MFGM and the fat core as they occur when the fat is totally liquid before freezing cannot be found. This distinct difference indicates that the binding forces between the outermost fat layer and the MFGM are generally higher than between individual monolayers of crystallized fat.

Fresh milk not only contains membrane-enveloped fat globules but also lipid-containing particles, which cannot be removed by cream separation but which sediment in a strong centrifugal field as a fluffy layer on the casein pellet (39, 42). These particles are membrane-coated, liposome-like vesicles, up to approximately 0.5 μm in diameter and are supposed to originate either directly from secretory plasma membranes or to have been partly released from milk fat globules. Fig. 27 demonstrates that such vesicles are usually cleaved within their membrane thus exhibiting numerous 'membrane particles'. In this respect they resemble membrane-coated vesicles within cells.

Finally it should be mentioned that the different fracturing characteristics of liquid and of crystallized fat facilitate the structural analysis of continuous fat phases such as in water-in-oil dispersions (14). The electron microscopic evaluation of size, shape, and spatial distribution of crystallized fat aggregates is important for improving physical properties such as firmness and spreadability in systems like butter (43-45).

6. Freeze-etching

Only those structural details which are directly situated in the plane of cleavage can be made visible by freeze-fracturing. The aim of freeze-etching is to make visible structures located below this plane. Of course, only sufficiently volatile substances can be sublimed at low temperatures. Normally, substantial sublimation rates are only obtained with frozen water.

The recession of ice is exponentially related to temperature and equals approximately 2 mm/sec at 173 K and 0.2 mm/sec at 163 K (35, 61). For obtaining reproducible freeze-etching conditions, a cold trap (e.g. the knife of the freeze-microtome in the Balzers unit) should be placed as near as possible to the freeze-fractured surface of the specimen and the vacuum should be as high as possible.

Only limited sublimation of ice from an aqueous specimen is possible if a cryoprotective treatment has been necessary to prevent freezing artefacts. Because of the low vapor pressure of glycerol, only small amounts of water can be removed from the freeze-fractured surface of such samples. Fig. 28 (skim milk cryoprotected with 33% glycerol) shows the effect of freeze-etching at 193 K for 5 min. This treatment would remove a 20 μm thick layer of pure ice. However, only a slight recession of the serum phase can be seen. The original plane of cleavage corresponds to the location of the cross-sectional areas of the casein micelles. Several smaller micelles which had been situated directly below this plane are now visible as small smooth elevations.

It is obvious that deep etching of aqueous samples can only be performed when they can be cryofixed in the absence of cryoprotectants. Only pure distilled water or very dilute buffers (less
The fracturing characteristics of crystallized fat globules are mainly determined by the spatial arrangement of crystal layers within a globule. In homogenized systems, peripheral fractures within the predominantly concentric crystal layers occur almost exclusively. Fig. 24 shows a low-fat dairy spread with convex (1) and concave (2) fracture views. The aqueous phase of this specimen exhibits freezing artefacts due to insufficient penetration of glycerol. Fig. 25 and 26 are of a cooled unhomogenized cream (for buttermaking) which not only shows peripheral fractures (1: convex; 2: concave views) but also cross-fractures (3). Bars in Fig. 24 and 26, 1 μm; bar in Fig. 25, 5 μm.

Fig. 27 (facing page, right column). Small lipid vesicles (V) in skim milk are mainly fractured within their membrane (similar to biomembranes) and thereby exhibit membrane protein particles. (IC: interior fracture face; EP: eutectic phase). The occurrence of very small casein micelles (C) is the result of the centrifugation of the skim milk. Bar, 0.5 μm.

Fig. 28 (facing page, right column). Cryoprotecting a skim milk sample with 33% glycerol allows limited sublimation of ice. This preparation resulted from etching at 193 K for 5 min. Cross-fractured casein micelles (1) now appear slightly raised above the serum level while nonfractured micelles (2) appear as smooth elevations. Bar, 0.5 μm.

Fig. 29 (facing page, right column). Extensive views of the outer surface of fat globules are obtained by freeze-etching 'washed' cream specimens (in distilled water). This view of a fat globule from freshly secreted cows' milk has been obtained after freeze-etching at 163 K for 5 min. In the center of the globule the outermost layer of the fat core (φ) is exposed as a result of fracturing. Arrows indicate the beginning of the fat globule membrane (M) which has been made visible by subliming the ice phase. The outer surface of the fat globule membrane appears to be covered either sparsely (1) or densely (2) with granular material. Bar, 1 μm.

than 10 mM) should be used, in order to avoid artefacts caused by a deposition of dissolved material (33). Although aqueous systems generally have to be cryofixed by ultrarapid freezing techniques such as the spray-freezing technique have to be applied (2-5). We have used this method for studying the temperature-dependent aggregation of β-casein (16) and for visualizing α-lactalbumin and β-lactoglobulin molecules (50). These globular proteins are hardly detectable in freeze-fractured preparations (in contrast to the randomly coiled caseins) but exhibit a diameter of approximately 15 nm in a freeze-etched preparation (see Fig. 35). The differences in size between freeze-fractured and freeze-etched protein particles have to be ascribed to the fact that during freeze-fracturing the particles are severely damaged, i.e., mostly torn apart, whereas they are completely set free during the etching process.

Furthermore, the fine network of gel systems can be made visible, because freeze-fracturing alone cannot provide the necessary information in the third dimension. The application of ultrarapid freezing methods is essential for such highly hydrated systems (17, 23). As examples, the heat-coagulated soy protein gel (Fig. 9-11) and the sol-gel transformation of gelatin (Fig. 12 and 13) can be taken.

The reason for the slightly aggregated state of the particles in the gelatin sol (Fig. 12) is not yet known, but it could well represent beginning gelation due to a temperature drop during handling before cryofixation. But it could also represent an artefact since protein molecules and virus particles can rearrange into larger aggregates during very deep etching (30).

In this context it should be mentioned that Hood and Allen (24) freeze-etchcd carrageenan-milk gels in the presence of 20% glycerol, which was not high enough to prevent freezing artefacts but which enabled the occasional detection of fibrils which were supposed to represent the gel network. Generally, various types of etching (and condensation) artefacts can occur in glycerinated samples (9, 10, 56). Fig. 30, 31, and 32 illustrate that pits of varying sizes and shapes can appear as the result of random or controlled etching. It is also noteworthy to realize that protein molecules which have been deformed during freeze-fracturing are able to partially restore their original structure during freeze-etching. This has been demonstrated with catalase crystals (30). Very dense protein particles as they are present in natural hard cheese varieties or in process cheese exhibit a considerably finer particulate structure after freeze-etching, which results in a slight sublimation of ice, as compared with
Fig. 30, 31, and 32. Various types of artefactual etching patterns may occur in glycerinated aqueous phases. Fig. 30 shows a solution of β-casein (see also Fig. 1) where a short uncontrolled etching occurred at 163 K and produced small depressions (D) of approx. 50 nm in diameter. P: protein particles. Fig. 31 (an emulsion of butterfat in whey) and Fig. 32 (whole milk) are of freeze-etched preparations (173 K, 1 min) of cryoprotected (33% glycerol) systems, which exhibit an uneven sublimation of ice. F: parts of cross-fractured fat globules. Bars, 0.5 μm.

freeze-fractured preparations (Fig. 33 and 34). However, the structural differences between different types of cheese are easier to detect in un-etched preparations, as already mentioned in Section 5. In earlier freeze-etch studies of Cheddar, Cheshire, and Gouda cheese (21), differences between the protein matrices were reported but these specimens had been cryoprotected with glycerol whereas those shown in Fig. 33 and 34 were not.

Finally it should be mentioned that media other than water allow a controlled sublimation. It is demonstrated in Fig. 17 that dioxane sublimes under conditions which are somewhat similar to those for water.

7. Replication

To obtain the highest amount of information about the fine structure of freeze-fractured/freeze-etched food samples (as well as about any other type of sample), high quality replicas have to be made. Guidelines for the performance of the replication step and for the critical evaluation of the quality of the replicas have been published repeatedly (25, 36, 57, 59, 61).

Although unidirectional shadowing is normally most suitable for replication, the adaptation of rotary shadowing to freeze-fracturing/freeze-etching has resulted in an improved presentation of subunit structures of protein particles on biomembranes (31). Recently we applied this technique to molecular aggregates (submicelles) of casein which had been spray-freeze-etched in a dilute buffer in order to study the true size of these particles. Fig. 35 shows unidirectionally shadowed β-lactoglobulin molecules, and in Fig. 36 rotary-shadowed casein submicelles are shown. The high contrast at the periphery of the rotary-shadowed particles allows more precise measurements of size and shape to be made than on unidirectionally shadowed preparations. However, the interpretation of rotary-shadowed specimens is impaired by the observation that the appearance of small particles such as ferritin molecules varies dramatically depending on the shadowing angle and the thickness of the metal film (38). The apparent size of unidirectionally shadowed protein particles in freeze-etched preparations, measured perpendicularly to the direction of shadowing, has to be reduced by approximately twice the thickness of the deposited heavy metal film (generally 1.5-2 nm) in order to obtain the true diameter of the hydrated particle (52). Although the quality of the shadowing layer largely determines the resolution of the replica, the additionally deposited layer of pure carbon (10-30 nm) can result in peculiar contrast
characteristics at the periphery of free particles (53).

Finally it should be mentioned that occasionally it is favorable to tilt the freeze-fracture/freeze-etch replica in the electron microscope in order to obtain an optimum contrast locally (60). This is especially valid for systems in which the fracture plane exhibits great spatial variation, such as in crystallized fat phases (44). In addition, it can be advantageous to take stereo pairs from a freeze-fracture/freeze-etch replica in order to obtain maximum information on the spatial structure (59). The reliability of interpretations, especially of very fine structures such as protein particles, can be considerably improved by applying double-fracturing techniques in order to obtain complementary freeze-fracture/freeze-etch replicas (59). This special technique has been repeatedly used for biological systems, especially membranes, but so far not for food systems.

Fig. 33 and 34. Two views of the protein matrix of a non-cryoprotected process cheese sample, one obtained after freeze-fracturing (Fig. 33) and the other view obtained after freeze-etching at 183 K for 1 min (Fig. 34). Protein particles are easier to identify in unetched preparations. Bar, 0.5 μm.

Fig. 35 and 36. Spray-freeze-etched (163 K, 5 min) preparations of β-lactoglobulin (Fig. 35) and casein submicelles (Fig. 36) in highly diluted buffers. The former sample was shadowed unidirectionally at an angle of 45° and the latter sample was rotary-shadowed at 25°. (L: latex sphere, added to the solution as a label.) Rotary shadowing appears to be especially suitable for evaluating the size and shape of small particles. Bar in Fig. 35, 0.5 μm; bar in Fig. 36, 0.1 μm.

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References


Discussion with Reviewers

M. Kalab: Dispersion of aqueous gelling systems in oil for freeze-fracturing by the oil-emulsion freezing technique is achieved using emulsifiers. Has it been established that such emulsifiers do not affect the microstructure of the aqueous gelling systems under study?

Author: According to our experience an oil-soluble emulsifier does not affect significantly the microstructure of the aqueous gelling system, especially if the (protein) concentration is high. At low concentrations (e.g. 1% or less) the gel formation could perhaps be adversely influenced by the adsorption of protein at the oil-water interface because this adsorption results in a decrease of protein in the serum phase. In the presence of an emulsifier in the oil phase, a reduced adsorption of proteins is to be expected.

M. Kalab: Platinum has been used most frequently for shadowing but some other metals, particularly tungsten, have been known to produce replicas of a lower granularity. What is your experience in this respect?

Author: In the past we have made a few experiments with the evaporation of tungsten/tantalum but found that for routine work the platinum/carbon shadowing provides a sufficiently high resolution and easier handling during cleaning of the replicas.

D.G. Schmidt: In the section entitled 'Chemical fixation' a peculiar pH effect is noted when (milk) proteins are fixed with glutaraldehyde. The pH drop suggests that during fixation protons are liberated and, therefore, the protein concentration should be of importance. Has the author noted such a concentration effect on the pH drop?

Author: Yes. There is a pronounced correlation between the pH drop and the protein concentration.

U.B. Sleytr: Have you any experience with freezing non-cryoprotected, highly hydrated gels on cold surfaces (e.g. using the van Harreveld and Cromwell technique)?

Author: No. I am not aware of any application of this technique for gels elsewhere.