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ELECTRON MICROSCOPY OF MILK AND MILK PRODUCTS: PROBLEMS AND POSSIBILITIES

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Abstract

Milk and dairy products have frequently been studied by transmission- and scanning electron microscopy. The specimen preparation procedure may considerably influence the final result, and formation of artefacts is frequently observed. In this respect, formation of ice crystals during cryofixation is a well-known phenomenon. But dehydration, to an extent such as is required for embedding procedures, also appears to be harmful to dairy products. Micrographs of thin sections of plastic-embedded samples of casein submicelles show threadlike material, whereas in freeze-etched specimens only spherical particles are found. Similar observations are made when samples of cheese and of concentrated milk are investigated. It is therefore concluded that the use of organic solvents for dehydration purposes is to be avoided when studying the fine structure of casein. High-voltage electron microscopy has not yet found any application to speak of in dairy research, but may become of interest in the study of the three-dimensional networks in milk gels by using thick sections. As yet electron microprobe analysis has found only little adoption in dairy research, viz. in energy-dispersive X-ray microanalysis of the calcium and phosphorus contents of casein micelles, and of the composition of crystalline inclusions in cheese.

Introduction

The direct way to observe microstructures is by microscopy. This is the way in which the microstructure of many food products including dairy products has been successfully investigated. Although interesting results have been obtained with light microscopy, only the application of electron microscopy with its much higher resolution has given a good insight into the microstructure of foods.

The first application of electron microscopy in dairy research was that by Nitschmann (46) who in 1949 investigated casein micelles in skim milk. Hostettler and Imhoff (35) made an electron microscopical study of milk and various dairy products. More recent reviews are those by Brooker (10) who studied selected dairy products by transmission electron microscopy of thin-sectioned specimens, by Kalab (37) who made a scanning electron microscopical investigation of a number of products and by Buchheim (11) who studied milk-, cream- and whey powders by transmission electron microscopy.

Electron microscopical techniques which have found application in dairy research have been extensively reviewed by Kalab (38) in 1981 and, as far as freeze-fracturing and freeze-etching are concerned, by Buchheim (12) in 1982.

In this paper special features of these techniques when applied to dairy products will be discussed and some techniques which are not covered by the reviews mentioned will be described.

Experimental techniques

A sample which is to be analysed in an electron microscope, where a high vacuum is present, should not contain volatile matter. Biological specimens, to which milk and milk products also belong, may contain water in quantities ranging from a few percent up to almost 100%. This means that the water vapour pressure of the sample must be sufficiently lowered before it can be studied in the microscope.

From the instrumental point of view, dehydration of the specimen is the most simple
Dehydration, however, is a severe operation as far as maintaining the initial microstructure is concerned, particularly when the water content is high. Therefore precautions have to be taken to avoid possible deteriorating effects of the dehydration.

From the specimen point of view the sample should be fully hydrated and the water vapour pressure should be lowered by cooling to such a value that the vacuum is not seriously affected. For this purpose the specimen must be cooled down to the temperature of liquid nitrogen or even still lower and it is thus studied in the frozen hydrated state. Reaching this state of the specimen without causing structural damages due to formation of ice crystals is not simple, but several approaches have been developed. In addition the instrumentation becomes more complicated, including a cold specimen stage in the microscope and sometimes a differential pumping system, allowing the vacuum in the immediate vicinity of the specimen to be lower than elsewhere in the column.

Two types of electron microscope may be distinguished, each with its own advantages and disadvantages. In the transmission electron microscope (TEM) the image is formed by the electrons which pass through the (partly) translucent specimen. In the scanning electron microscope (SEM) the primary beam is scanning the specimen. This gives rise to the emission of secondary electrons which are used for the image formation. The scanning principle may be combined with a TEM into a scanning transmission electron microscope (STEM). Since each point of the specimen will be exposed to the electron beam for only a fraction of the whole observation time, radiation damage may be reduced considerably.

Fig. 1. Air-dried casein micelles in skim milk. The milk was fixed with 2% formaldehyde for 1 h, 500 times diluted with distilled water, sprayed on formvar-coated grids and air-dried. Shadowing with Pd. Compare the triangularly shaped shadow of the collapsed micelles with the elliptical shadow of the undisturbed polystyrene latex sphere.

Fig. 2. Freeze-dried casein micelle in skim milk. The milk was fixed with 2% glutaraldehyde for 1 h and 500 times diluted with distilled water. A drop of the solution was placed on a formvar-coated grid, frozen in liquid nitrogen and freeze-dried. Shadowing with Pt/C. The roughly elliptical shape of the shadow indicates that the micelle has not collapsed.

Fig. 3. Freeze-etched casein micelle in skim milk. The milk was fixed with 2% glutaraldehyde for 1 h, 100 times diluted with distilled water, cryofixed by Bachmann’s spray-freezing technique, freeze-etched at -110 °C for 6.5 min and shadowed with Pt/C. During etching a small micelle (arrow) has fallen on top of the big one (62).
A modern electron microscope is not only an image-forming instrument with a high magnification but a powerful analytical tool as well. The interaction of the electrons of the primary beam with the atoms in the specimen gives rise to the emission of X-rays and so-called Auger electrons, which are both characteristic for the atoms present. Analysis of these emissions by means of X-ray micro-analysis and Auger spectrometry respectively would thus yield information concerning the chemical composition of the specimen. The primary beam may be focused on the specimen to a very small spot so that the generated X-rays and Auger electrons originate only from a small region of the specimen. This makes it possible to analyse separate details in the specimen, independent of the surrounding matrix. Electron microprobe analysis may be carried out in a SEM as well as in a STEM.

Transmission electron microscopy

For transmission electron microscopy the specimen must be at least partly translucent for the electrons of the primary beam, which implies that the specimen must be very thin, of the order of 100 nm or less. The ways in which such preparations can be made in the case of dairy products have recently been reviewed by Kalab (38).

Suspensions of small particles such as casein micelles may well be studied by spraying on formvar-coated grids. In order to enhance the contrast in the specimen it may be stained negatively, for instance with potassium tungstate or uranyl acetate (23) or it may be shadowed using a heavy metal (1, 30, 67, 70). In order to avoid overlapping of the particles on the specimen grid, in most cases the suspension has to be diluted. Casein micelles, however, cannot be diluted with water without causing disintegration (46) and therefore have to be fixed, for instance with formaldehyde (46) or glutaraldehyde (13). After simple air drying and subsequent shadowing, which is mostly done with Pb/C, the micelles, in spite of their fixation tend to become flattened. This has already been observed by Nitschmann (46) and is well illustrated by Fig. 1. The collapsed casein micelles show a triangularly shaped shadow whereas the polystyrene latex particle shows the characteristic elliptical shadow of a sphere. In the case of negatively stained micelles such a flattening is not observed, probably because the particles are more or less completely embedded in the surrounding stain. The collapse of the micelles must be ascribed to the strong interfacial forces which occur when, during air drying, the receding water surface passes over the particles. When the micelles are freeze-dried, such a collapse is not observed (Fig. 2), which would imply that the interfacial forces at the solid–gas phase boundary are less harmful than those at the solid–liquid boundary. Also by freeze-etching, which is merely a particular form of freeze-drying casein micelles do not collapse.

For samples larger than colloidal particles and also for the study of the internal structure of such particles, for instance of the substructure of casein micelles, or of the degree of crystallization in fat globules, other techniques have to be applied. One of the most widely spread techniques is thin sectioning of plastic embedded samples (27). In this technique the sample is first properly fixed, for instance with glutaraldehyde and/or OsO₄ (25), subsequently dehydrated in a graded series of ethanol or acetone and embedded in some suitable plastic monomer, for instance araldite or epon (26). After hardening thin sections are cut using an ultramicrotome (27), and these sections may be post stained, for instance with lead citrate or uranyl acetate (28). Suspensions may be embedded by using Salaye's microparticle technique (54), in which the suspension is encased in small agar capsules, which are fixed, dehydrated and embedded just like a piece of tissue (34). This technique has been applied to the study of milk (31), homogenized milk (32), concentrated milk (21) and curd formation (33).

In this procedure the dehydrating and embedding steps are the most critical. During dehydrating the polar aqueous medium with a high dielectric constant is gradually replaced with a non polar organic medium with a low dielectric constant. This will influence both hydrophobic and electrostatic bonds in proteins and denaturation may occur even in fixed samples, and in particular with caseins, which have a more open structure than compact globular proteins and which have a high hydrophobic character (59, 62). This is well demonstrated by Fig. 4 in which fixed embedded casein submicelles show a thread-like structure whereas in freeze-etched specimens of spray-frozen solutions of these submicelles only small spherical particles are visible, Fig. 5. Also in negatively stained and shadow-cast preparations only spherical particles are observed (57).

In thin sections casein micelles mostly appear as circular cross sections, which due to compression effects during cutting may be deformed elliptically, Fig. 6. Their submicellar structure, which is easily revealed in freeze-fractured specimens, Fig. 7, can hardly be detected in this way. This may at least partly be ascribed to overlap of the submicelles in those sections of which the thickness is larger than the submicellar diameter. During dehydration denaturation of the submicelles may also have occurred, which might result in some swelling and would make the individual submicelles no longer visible in the sections. An indication of such a swelling of submicelles has been found with the electron microscopy of cheese, see below.

The milk fat in dairy products may sometimes give rise to problems during embedding. Lipids may be fixed by OsO₄ which reacts with double bonds in unsaturated fatty acids (25). Since the fatty acids in milk fat
Fig. 4 Plastic-embedded casein submicelles. The casein submicelles (obtained by dialysing skim milk against 0.07 M imidazole-HCl-NaCl buffer, pH 6.7) were encapsulated in agar microcapsules according to Salyaev, fixed with 1 % OsO₄ for 24 h, dehydrated with ethanol and embedded in styrene-methacrylate. Post-staining with lead citrate.

Fig. 5 Freeze-etched casein submicelles. The solution was cryofixed by Bachmann's spray-freezing technique, freeze-etched at -110 °C for 6.5 min and shadowed with Pt/C. Compare the spherical shape of the particles with the thread-like structures in Fig. 4.

Fig. 6 Plastic-embedded casein micelles in skim milk. For specimen preparation, see Fig. 4. The direction of sectioning is indicated by an arrow.

Fig. 7 Freeze-fractured casein micelle in skim milk. The milk was cryofixed by Bachmann's spray-freezing technique, fractured at -110 °C and immediately shadowed with Pt/C without etching. Note the submicellar structure of the micelle which is not visible in the plastic-embedded micelles, Fig. 6 (62).
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are for the larger part saturated, fixation is expected to be poor. This is clear in thin sections of araldite- or epon-embedded fat globules which frequently have a wavy appearance due to periodic compression of the fat tissue, which is softer than the surrounding resin matrix (38). With styrene and methacrylate as embedding media we sometimes observed considerable fat extraction in samples of whole milk and concentrated whole milk and increased hardening times as compared to skim milk samples.

A disadvantage of thin sections is the fact that in the case of extended three-dimensional structures such as for instance whole cells, bacteria or networks such as in curds and yoghurts only a cross section is obtained. A better idea of their actual structure, particularly in that of three-dimensional networks may be obtained by using sections with a thickness of the order of several μm. The study of such thick sections requires a higher penetrating power of the electron beam than is attainable with a normal 100 kV instrument. Therefore a high voltage electron microscope using acceleration voltages of 1 MV or more becomes necessary (36). In most cases the images thus obtained are rather complex and difficult to interpret. Interpretation may be much improved by the application of stereo microscopy in connection with these thick sections. Until now high voltage electron microscopy has found only limited use in biology (19).

Cryofixation, which is a pure physical process, may be used instead of chemical fixation. In this way the possible formation of artefacts due to the introduction of chemicals is avoided. Also substances such as polysaccharides, which are hardly fixed by glutaraldehyde or OsO₄ and which may be influenced by dehydration and embedding (25), can be studied in this way. With cryofixation, however, one must be aware of artefacts due to the formation of ice crystals which in suspensions will lead to phase separation and an uneven distribution of the particles, and

Fig. 8 Freeze-dried cryo section of Gouda cheese. The cheese was cryofixed by immersion in melting N₂ and thin-sectioned using a specimen temperature of -100 °C and a knife temperature of -80 °C. The section was freeze-dried and contrasted with OsO₄ vapour.

Fig. 9 Freeze-fractured Gouda cheese. The cheese was cryofixed by immersion in melting freon, fractured at -120 °C and immediately shadowed with Pt/C without etching.

Fig. 10 Plastic-embedded Gouda cheese. The cheese was fixed with 1% OsO₄ for 24 h, dehydrated with acetone and embedded in araldite. Post-staining with lead citrate.

F: fat globule, S: submicellar-like particles. Compare these particles with those in Figs. 8 and 9.
in tissues and similar specimens to structural damages due to expansion of the freezing water. In order to reduce formation of ice crystals to a level where they are no longer harmful, freezing must be carried out very rapidly, which implies that only small specimens can be handled, or some cryoprotectant has to be added (50, 66). The subjects which are important in this respect such as ice nucleation, vitrification and recrystallization have been extensively discussed by Franks (20) and shall not be dealt with again.

For examination in the electron microscope the cryofixed specimen may be dehydrated in different ways. Freeze-drying is a simple way but requires that possible cryoprotectants are volatile or have not been added at all (50). Dehydration may also be accomplished by removing the ice with organic solvents which is done during freeze substitution (50). To this end the frozen specimen is immersed in a solvent such as acetone or dimethyl formamide at -80 °C. Complete substitution may take up to two weeks. Then the substitution fluid is changed, after which the temperature is raised. With dairy products, however, one has to be cautious because of casein denaturation which may alter the protein fine structure as has been mentioned above. In addition the solvents used may extract lipids from the specimen. The third method is that of freeze replication which is done with freeze-fracturing and freeze-etching, techniques which are discussed by Buchheim elsewhere (12). Finally the cryofixed sample may be thin-sectioned using a cryomicrotome (65). The sections thus obtained may be studied either after freeze-drying or directly in the frozen hydrated state, which requires that the microscope is equipped with a cooled specimen stage and a specimen transfer unit (24, 71).

We have applied the cryo thin-sectioning technique for the study of Gouda cheese in the following way. A small piece of the cheese (1 mm³) is cryofixed by immersion in melting nitrogen and subsequently transferred to a LKB ultramicrotome equipped with a cryochamber. In biological tissues with a high water content only the outer 5 to 10 µm of the specimen freezes without significant formation of ice crystals (64). In cheese, where the water content is about 40 %, which water is for a large part bound to salt ions as hydration water, this layer may be thicker. Sectioning is carried out with a dry knife using a speed of 2 mm/s; the temperatures of knife and specimen are -80 and -100 °C respectively. The frozen sections are collected on formvar-coated grids and flattened by pressing as described by Sevée (64). After freeze drying the sections are contrasted by exposure to O₂O vapour. Fig. 8 shows the protein microstructure in cheese revealed by this technique. The protein matrix consists of a continuum of submicellar-like particles in which the fat globules are embedded. The latter, however, are apparently poorly fixed and tend to melt during observation thus locally obscuring the submicellar structure.

This particle structure of the protein matrix is well comparable to that observed in freeze-fractured samples. The size of the particles in the plastic-embedded sample is about 20-40 nm, which is twice as much as that in cryosectioned and freeze-fractured preparations. This might be caused by a swelling of the casein during dehydration when there is a transition from a polar to an apolar medium.

Another discrepancy is observed with sterilized concentrated milk. In thin sections of plastic-embedded samples filamentous structures can be distinguished, which protrude out of the casein micelles, Fig. 11 (2, 5, 21). In freeze-fractured specimens, however, only a large number of free submicellar-like particles can be detected (30). In shadow-cast preparations only small particles or short chains of such particles are observed (55, 56). In concentrated milk, sterilized at ultra high temperatures a gelation, designated as age-thickening (22) is frequently observed during storage. Thin sections of plastic-embedded samples show a strong increase of thread-like structures between the micelles, Fig. 13 (2, 21). In freeze-fractured specimens, however, such threads are not observed, Fig. 14 and in shadow-cast specimens threads are not observed either (55, 56). Instead of threads an increased number of free submicellar-like particles is observed. The thread-like structures in thin sections of plastic embedded specimens may therefore be artefacts rather than actual structural features. These artefacts may have been caused by an unfolding of free casein submicelles and of small aggregates of heat-denatured whey proteins under the influence of the organic solvents used for dehydration as explained above.

Scanning electron microscopy

In scanning electron microscopy an image is obtained of the surface of the specimen, which therefore needs not to be a thin one. In fact rather bulky samples are frequently studied in a SEM.

In food research most samples are non-conductive for electrons and in order to prevent charging of the specimen, which might result in a distortion of the image, the electrons of the primary beam must be eliminated in some way or another.

In most cases the surface is made conductive by deposition of a carbon or metal layer on the specimen, which may be done by vacuum evaporation or by sputter coating. The use of a heavy metal such as gold is to be preferred over that of carbon because of the higher yield of secondary electrons for image formation. Sputter coating results in a more continuous surface layer than does vacuum
Fig. 11 Plastic-embedded concentrated milk.
For specimen preparation, see Fig. 4.
F: fat, C: casein.

Fig. 12 Freeze-fractured concentrated milk.
For specimen preparation, see Fig. 7.
F: fat, C: casein.

Fig. 13 Plastic-embedded concentrated skim milk which has gelled during storage.
For specimen preparation, see Fig. 10.

Fig. 14 Freeze-fractured concentrated skim milk which has gelled during storage.
For specimen preparation, see Fig. 9.

Evaporation, particularly with specimens with large height differences, is a concern. The thickness of the metal layer limits the resolution which might be obtained. The specimen may also be damaged due to the heat which is developed during the coating process. Such complications can be avoided by making the whole sample conductive for electrons by the application of conductive staining or metal impregnation procedures. These methods, which are mostly based on a treatment of the specimen with OsO₄, and tannic acid and/or thiocarbohydrazide, have been extensively reviewed by Murphy (44, 45). Such methods, however, have not yet found application in dairy research.

The specimen may also be observed in the frozen hydrated state, and provided that sufficient ions are present, no charging will occur (41). However, in preliminary experiments with cheese we observed a considerable charging...
Fig. 15 Cryo-scanning electron micrograph of Gouda cheese. The cheese was cryofixed by immersion in melting N2, fractured at -100 °C, coated with carbon and observed at -100 °C. F: fat globule, C: casein, M: fat globule membranes (60).

Fig. 16 Cryo-scanning electron micrograph of whipped cream. For specimen preparation, see Fig. 15. A: air bubble, F: fat globule (61).

of the specimen, which could be suppressed by deposition of a carbon layer on the specimen (60).

In water-containing products the water must be removed by drying or the sample must be cooled for a sufficient lowering of the vapour pressure. To this end the same procedures may be applied which have been described in the previous section for transmission electron microscopy, albeit that the practical performance may be somewhat different.

A technique which has found much application in scanning electron microscopy is that of critical point drying, by which the strong interfacial forces occurring at phase boundaries are circumvented (15). The method is based on the fact that when the temperature and pressure of a liquid are increased, a point will be reached where liquid and gas are indistinguishable. At this so-called critical point the surface tension of the liquid is zero. By going around the critical point, thus circumventing a crossing of the liquid-gas phase boundary, it is possible to reach the gaseous state from the liquid state without the high surface forces which result from receding phase boundaries. Unfortunately the critical point of water, 374 °C at 22.5 MPa is far too high for most biological applications. The water therefore has to be replaced by a more appropriate liquid such as carbon dioxide or Freon 13 with critical points at 36.5 and 28.9 °C and 7.6 and 3.9 MPa respectively. Since these liquids are immiscible with water, the specimen must first be dehydrated with ethanol, which on its turn is replaced by amyl acetate and finally by carbon dioxide. In the case of Freon 13 the amyl acetate is omitted. Because of the use of organic solvents, however, artefacts may be expected with respect to the casein fine structure. Substances which are less well preserved during fixation, such as fats and polysaccharides, may be extracted during the procedure.

For the latter substances cryotechniques in combination with a cryo specimen stage in the microscope seem more appropriate. One has to take considerable care, however, that artefacts due to formation of ice crystals do not occur. In this respect products with a relatively low water content are least liable to such artefacts. We have applied a rather simple cryotechnique to the study of cheese (60) and whipped cream (61) in which the sample was frozen in liquid nitrogen, fractured and coated with carbon in a freeze-etch unit and finally transferred to the cryostage of a SEM.

In the micrograph of cheese, Fig. 15, the fat globules are well preserved. Remnants of fat globule membranes are also visible. Their wrinkled appearance is probably caused by partial adhesion to the fat globules when they are broken away during fracturing. In whipped cream, Fig. 16, the air/serum interface of the air bubbles is well visible. It seems to consist of fat globules which are embedded in liquid fat, which has flowed out of the globules during whipping. It is clear, however, that in the serum phase some formation of ice crystals has occurred.

Other sample preparation techniques for scanning electron microscopy and their applications in dairy research have been described at length by Kalab (38).
Electron microprobe analysis

In the section on experimental techniques it was mentioned that an electron microscope can also be used as an analytical instrument for the determination of the chemical composition of the specimen. For this purpose one may use the X-rays as well as the Auger electrons which are emitted by the specimen under the influence of the primary electrons.

Due to inelastic collisions of the electrons of the primary beam with the atoms of the specimen, an electron of one of the inner shells may be ejected, which results in the formation of highly excited ions. These ions return to a lower energy level when an electron of one of their outer shells replaces the ejected electron. The difference in potential energy between the replacing and the ejected electrons is characteristic for each chemical element. The excess energy may be emitted as an X-ray photon which can be studied by X-ray microanalysis (51). The excess energy may also be dissipated by the ejection of another outer shell electron. These so-called Auger electrons are also characteristic for the atoms present and can be analyzed by an Auger electron spectrometer (14, 17).

Since the only detectable Auger electrons originate from a layer with a thickness of 1-2 nm below the surface of the sample, Auger electron spectrometry is a typical surface analysing technique. Even the slightest contamination of the surface will interfere with the analysis so that an ultra high vacuum is required (≤ 10^-5 Pa). As yet this technique has only found limited application, mostly in material sciences, and therefore it will not be discussed further.

X-ray microanalysis is a widely spread technique, not only for the study of materials but also for the analysis of biological samples, including foods. The technique has also found several applications in dairy research. The X-rays which are emitted by the specimen are characteristic for the chemical elements present since they are related to the energy levels in the atoms. A qualitative chemical analysis of the specimen may thus be given by identification of the different lines in the X-ray spectrum.

Although the intensity of the characteristic X-rays is related to the concentrations of the elements in the irradiated volume of the sample, a quantitative chemical analysis is difficult to perform because of several interfering phenomena (52). It must first be recognized that the X-ray emission takes place from the whole volume of the specimen which is influenced by the primary beam, Fig. 17. The primary electrons penetrate into the specimen and give rise to an emitting volume whose size and shape are determined by their kinetic energy and the composition of the specimen. The depth of penetration is mostly several μm; the lateral expansion which determines the resolution of the method is of the same order of magnitude in spite of the fact that the primary beam may be focused to a spot with a size of a few nm.

Serious interference is caused by deceleration and deflection of the electrons of the primary beam by the nuclei of the atoms. This gives rise to X-rays with energies ranging from 0 up to the kinetic energy of the incident electrons. This continuous radiation, frequently designated as "Bremsstrahlung" results in a background in the energy spectrum on which the characteristic emission is superimposed. Another complication for a quantitative analysis is absorption which depends on the composition of the specimen, the energy of the generated X-rays and the instrumental geometry. Absorbed X-rays may also introduce ionization of the inner shells of other atoms, thus giving rise to secondary X-rays. This so-called secondary fluorescence is, however, less important than absorption. Another source of interference are extraneous X-rays which are caused by the interaction of stray electrons, backscattered electrons and stray X-rays with the mechanical parts of the system such as pole pieces, specimen holders, etc. Finally it must be recognized that during analysis of the specimen in the microscope the specimen may lose mass due to evaporation, mainly but not exclusively, of light elements. On the other hand a gain in mass may result from contamination due to condensation of residual gas in the column on the specimen.

In order to meet these sources of interference in quantitative work, where absolute concentrations or concentration ratios are to be determined, it has therefore become the custom to apply standard samples for calibration. For the most accurate analysis it is desirable that the standards used resemble the specimen as closely as possible because the corrections for the effects mentioned above only cancel out for identical preparations. When such an ideal standard is impossible, so-called ZAF corrections, which take the effects of atomic number (Z), absorption (A) and secondary fluorescence (F) into account, have to be applied (43). Although such corrections were primarily worked out for metallurgical purposes they may also be used for biological samples where in most cases only the absorption correction appears to be significant (52). In the case of thin sections the standard needs not be ideal and several quantification procedures may be applied (53). In thick sections the quantification is more difficult when the standard differs much from the sample under investigation. The methods required for the quantification of such samples have recently been reviewed by Boekestein et al. (6).

Detection of the X-rays may be done in two different ways, energy-dispersive (ED) or wavelength-dispersive (WD), depending on the type of detector used. A description of these detectors, which falls outside the scope of this review, has been given by Reed (51). Both methods have their advantages and disadvantages. In an ED system all elements are detected simultaneously within a few minutes whereas in a WD system the elements have to be detected one after another and a complete analysis may...
take several hours. The energy resolution of a WD system is an order of magnitude better than that of an ED system, which results in less peak overlap. Finally it must be mentioned that the background in a WD spectrum is mostly so low that correction is relatively simple. In an ED spectrum the background contributes significantly to the peak intensities so that a substantial correction has to be applied. Special computer programs have been developed for this purpose.

Both methods have approximately the same sensitivity, about $10^{-18} \text{g}$ of the element under study may be detected in the irradiated volume, which in practice corresponds to a relative concentration of 0.1%. All elements beyond Se may be detected by WD analysis whereas in ED analysis all elements beyond Na are detected. The latter lower limit may be shifted downwards to C by using a so-called window-less detector, but in most cases this is of little use because of the low resolution of the ED method in the low-energy part of the spectrum. The spectral data may be expressed either as a net peak intensity or as a peak-to-background ratio. The latter method has the advantage of being relatively insensitive to variations in specimen thickness, to fluctuations in the beam intensity and to variations in the local geometry (7).

Specimen preparation for X-ray microanalysis

It is obvious that for an analysis of individual features of the specimen the different preparation steps should not lead to a redistribution of the elements to be investigated (16). Also material losses and gains must be avoided, in particular when they are selective rather than general. Soluble ions such as Na$^+$ or K$^+$ are more easily liable to redistribution and elimination than are insoluble salts such as calcium phosphate.

It is worthwhile to mention, however, that we were unable to detect any calcium phosphate in thin sections of casein micelles, which were fixed by OsO$_4$, dehydrated with ethanol and embedded in methacrylate. Although the micelles remained intact and the protein part might be well preserved, calcium phosphate had apparently been leached away. O'Brien and Baumrucker (47) observed that in air-dried untreated casein micelles calcium and phosphorus were still present. In glutaraldehyde-fixed micelles, however, the amounts of these elements had fallen to about one third of the values in the unfixed sample.

Specimens for X-ray microanalysis may be prepared by drying methods such as air-drying, freeze-drying or critical point drying, by embedding techniques or by freezing techniques. The application of drying may be expected to result in considerable redistribution, notably of the soluble elements in the specimen, unless they can be adequately fixed or rendered sufficiently insoluble by the addition of suitable precipitants (16). Insoluble material can easily be analysed as is demonstrated by the study of Blanc et al. (3, 4) who determined the composition of needle-like crystals in Gruyere cheese. These authors made a SEM study of this cheese, which was fixed with acrolein/
glutaraldehyde, dehydrated in ethanol, defatted in chloroform, post-fixed with OsO₄ vapour and coated with gold. In the crystals observed, Fig. 18, only calcium was found and almost no phosphorus from which it was concluded that the crystals were calcium tyrosinate rather than calcium phosphate. Although crystalline inclusions in cheese and processed cheese have frequently been observed in the SEM as well as in the TEM (9, 10, 49, 69) these have not been characterized further by X-ray microanalysis.

During air-drying some elements might become concentrated at the gas-liquid boundary whereas during freeze-drying this may happen at the gas-solid boundary. Dehydration using organic solvents, which is applied in critical point drying and freeze substitution may lead to translocation of components which are difficult to fix, such as polysaccharides and lipids and of the elements which are associated with them.

Embedding procedures are also liable to migration of certain components during the dehydration. Dehydration or drying may also result in redistribution of elements in particular when hard and soft regions occur side by side in the specimen. Also the tray liquid may give rise to redistribution or leaching.

Cryofixation might be expected to cause less redistribution and losses in the specimen. During freezing, however, eutectic phases may be formed; at first pure water crystallizes and the remaining solution becomes more concentrated, which process continues until the whole solution solidifies at the eutectic point and a considerable redistribution takes place. Not only do small ions migrate in this way but also macromolecules, casein micelles and even polystyrene latex spheres with a diameter of 100 nm may concentrate at the phase boundary (63). Such segregation phenomena may be reduced by increasing the freezing rate or by the addition of a suitable cryoprotectant (66).

The cryo-fixed sample is best analysed in the frozen hydrated state since any attempt to remove the water might result in redistribution or extraction as has been mentioned above. This requires that the microscope is equipped with a cold specimen stage and that precautions have been taken to avoid contamination of the specimen when it is transported from the preparation stage to the microscope. For scanning electron microscopy a so-called bio-chamber has been developed which is attached to the microscope and in which the specimen is cooled by liquid nitrogen (48). The sample is fractured and coated in the chamber and may be moved into and out of the microscope at will via an isolation valve. Also frozen hydrated sections may be transferred without contamination from the cryomicrotome to the TEM by using a special transfer unit (24, 71).

As has been mentioned in the section on transmission electron microscopy the ideally frozen part of the sample, where no artefacts due to ice crystal formation have occurred, is only a rather thin surface layer of the specimen. Not only the morphology of the specimen but also its chemical analysis must be studied in this superficial zone. If bulk specimen or features well below the sample surface have to be analysed the use of cryoprotectants therefore cannot be avoided (66).

Conclusions

For the electron microscopy of dairy products the techniques to be used depend on the goals to be reached. The use of organic solvents for dehydration purposes may lead to the denaturation of casein and formation of artefacts and should therefore be avoided when studying protein fine structures.

Thin sections of plastic-embedded samples are well suited for the study of the distribution of fat globules and bacteria in cheese and yoghurt and also for the networks formed by casein micelles in milk gels. These networks may even be better studied by high voltage electron microscopy of thick sections, which results in a high resolution which is an order of magnitude better than that attainable in scanning electron microscopy.

Cryofixation, followed by freeze-fracturing, freeze-etching or cryo thin-sectioning seems to be the best technique to study protein fine structures and substances such as fats and polysaccharides, which are only poorly fixed by chemical means. Precautions have to be taken to avoid formation of artefacts resulting from ice crystals which are formed during improper freezing procedures. The usable area of the specimens thus obtained is in general much smaller than that obtained by thin sectioning of plastic-embedded samples. These techniques are therefore less suited for studies at low magnification.

Cryo thin sections may become very important for the study of the protein fine structure of dairy products. The sections may be studied either in the freeze-dried or frozen-hydrated state. The first method is the least complicated and is for structural studies perhaps as good as the second one. The method can successfully be applied to all products with a low fat content or to specimens from which the fat has been removed.

For X-ray microanalysis an ED study is rapidly carried out as a first survey of the sample. For an accurate analysis, however, an ED system is more appropriate, in particular for the lighter elements C, N and O, where a WD system has a much higher resolution than an ED system.

The analysis of frozen hydrated specimens will in principle give the best information but the occurrence of redistribution of constituents during cryofixation must be carefully checked. Also material losses, particularly of water and material gains, due to condensation of residual gas into the cold specimen, must be accounted for.

For many investigations, such as the study of crystalline inclusions in cheese and processed cheese, sections of plastic-embedded samples or dehydrated bulk samples may give
satisfactory results, but one should be aware of material losses during preparation.

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Discussion with Reviewers

M. Kalab: It is interesting to see casein 
submicelles or the δ-casein-β-lactoglobulin 
complex in the form of minute globules in 
freeze-fractured preparations and in the form 
of filaments in thin sections. Were attempts 
made to pinpoint the stage during embedding 
when the unfolding takes place? Were milk 
samples impregnated with alcohol or resin 
monomers subjected to freeze fracturing? What 
were the results?

Author: We made some experiments in which 
casein micelles were fixed with glutaraldehyde 
or OsO₄, dehydrated with a graded series of 
ethanol and impregnated with methacrylate 
monomers. The micelles were subsequently 
returned into the aqueous medium via the 
reversed way. Freeze-fractured specimens of 
these micelles did not reveal any unfolding of 
the submicelles. This would indicate that, at 
least with submicelles, the unfolding is 
reversible. We do not yet have experience with 
heated systems in which denatured whey 
proteins are present.

D.C. Carpenter: Could the increased submicellar-
like particles in the freeze-fractured, sterile 
and concentrated milk specimens be casein-whey 
protein aggregates?

M.E. Green and B. Brooker: Severe heating of 
milk is reported to cause micelle enlargement 
and an increase in the amount of non-
sedimentable casein. Why then does the author 
think that the submicellar-type particles in 
stereilized, concentrated milks are not derived 
from casein micelles?

Author: The submicellar-like particles most 
probably are aggregates consisting of non-
micellar casein and denatured whey proteins. 
Their composition will not be constant and the 
ratio casein to whey protein in these 
aggregates may vary between wide limits. 
Particles consisting merely of pure casein 
or pure denatured whey protein may also occur.

Dairy Res. 46, 317-323) has recently proposed 
that casein micelles are hairy, i.e. that they 
have peptide chains protruding into the milk 
serum. Do you think that electron microscopy 
could provide further evidence for this concept? 
Which preparation technique would probably be 
suitable for revealing the hairy surface 
structure?

Author: If the concept of the "hairy micelle" 
is correct the hairs consist of polypeptide 
chains. The detection of such thin threads by 
electron microscopy is at the limit of the 
possibilities with the present techniques. In 
dried micelles the hairs would stick to the 
micellar surface and something similar would 
happen when the micelles are embedded in an 
apolar resin. Embedding in water-miscible 
resins such as durcupan or glycophenacylate 
might reveal such a hairy surface, but I am 
not optimistic. I consider the freeze-etching 
technique to be more promising. Dr. Buchheim 
and I made some preliminary experiments with 
freeze-fracturing of spray-frozen dispersions of 
casein micelles. The specimens were slightly 
etched (about 20 nm) and subsequently rotate 
shadowed at an angle of 10-25° with the
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horizontal plane. We did not obtain clear evidence for the existence of such hairs.

M. Rüegg: Cold storage causes profound changes in the distribution of various milk constituents. Because of the dissociation of casein and inorganic components from the micelle into the soluble phase one would expect a change in the structure and/or average size of the casein particles. Electron microscopical results reported in the literature are conflicting (Schmidt DG, Van der Spek CA, Buchheim W, Hinz A. (1974). Milchwissenschaft 29, 455-459; Schmutz M. (1980). Dissertation ETH Zürich, Nr. 6651, 48-51). Do you have an explanation for the discrepancy between the conclusions drawn from size-measurements on freeze-fractured milk samples?

Author: Schmutz made measurements in samples which were cryofixed by means of a propane jet freezer, whereas Schmidt et al. mixed the sample with glycerol after which it was cryofixed by immersion in liquid freon. The presence of glycerol may influence the measured distribution. On the other hand Schmutz did not apply a correction for the facts that the plane of fracturing generally does not pass through the centre of the particles and that large particles have more chance to be fractured than small particles. We have the experience that small variations in the apparent size distribution frequently result in large variations in the actual size distribution.

W. Buchheim: You have demonstrated that freeze-dried and stained cryo-sections of Gouda cheese give a very detailed view of the protein matrix. Can you already estimate the applicability of this method to other types of dairy products? Does the water content of a sample play a similar critical role as it is the case during cryofixation for freeze-fracturing?

Author: Cryo thin-sectioning can be applied to all types of dairy products. The technique will be particularly useful for the study of milk gels such as curds and yogurt and, provided that a cooled specimen stage is available in the microscope, for the study of high-fat products such as creams. Also for electron probe microanalysis cryo thin-sectioning in combination with a cold specimen stage is the obvious technique. The water content of the sample is of much importance. With a low water content large areas of the sample will be well preserved during cryofixation, but when the water content is high only the thin outer layer of the sample will be frozen free from artifacts due to the formation of ice crystals. I think that one has to put up with this because the alternative, the application of a cryo protectant such as glycerol, may even be worse in most cases.

B. Blanc: Heat treatment of milk affects rennet-coagulation. In some cases the micelles do not coagulate at all. Does electron microscopy reveal any structural features which could explain the different behaviour of native and heat-treated casein micelles?

Author: Severe heat treatments will result in precipitation of whey proteins on the micelles and complex formation between \( \alpha \)-casein and \( \beta \)-lactoglobulin, which thus influences the rennet coagulation. The detection of the precipitated \( \beta \)-lactoglobulin is almost impossible unless the \( \beta \)-lactoglobulin could be suitably labelled so that the precipitated protein could be actually observed in the electron microscope. I do not know such a labeling technique. The gold labeling, applied by Schmidt and Both (Schmidt DG, Both P. (1982). Milchwissenschaft 37, 336-337) for the location of casein components in casein micelles cannot be used for the globular \( \beta \)-lactoglobulin molecules since the latter would easily be denatured during the labeling process.