Food Structure

Volume 7 | Number 1 Article 13

1988

Sample Holders for Solid and Viscous Foods Compatible with the Hexland Cryotrans CT 1000 Assembly

Miloslav Kalab

Paula Allan-Wojtas

Ann Fook Yang

FOOD MICROSTRUCTURE, Vol. 7 (1988), pp. 115-120
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0730-5419/88$3.00+.00

SAMPLE HOLDERS FOR SOLID AND VISCOUS FOODS COMPATIBLE WITH THE HEXLAND CRYOTRANS CT 1000 ASSEMBLY

Miloslav Kaláb, Paula Allan-Wojtas, and Ann Fook Yang*

Food Research Centre and *Plant Research Centre
Research Branch, Agriculture Canada, Ottawa, Ontario, Canada K1A OC6

Abstract

A brass block, 3.7 mm high and 10 mm in diameter, which has three openings to accommodate rivet-type or plain tubular specimen holders for scanning electron microscopy at low temperature, has been designed to fit the Hexland Cryotrans CT 1000 assembly in place of the original aluminum disc. Viscous food samples are placed in two-piece tubular holders (0.9 mm inner diameter, 1.2 mm outer diameter) made from sterling silver, and rapidly frozen. The holders are inserted into the brass block under liquid nitrogen and tightened with individual setscrews. A handle screwed into the central hole of the block facilitates manipulation of the block. The samples are fractured inside the Cryotrans CT 1000 assembly by knocking off the part of the sample located in the upper tube. The subsequent operations are the same as those suggested by Hexland.

A second simple type of holder has been developed for low-moisture foods, such as cheese, which are resistant to ice crystal formation during freezing. This holder consists of a Hexland aluminum sample disc drilled with a single opening (4.0 mm in diameter) temporarily closed at the bottom with sticky tape. The food is sampled with a cork borer and the sample plug is then inserted into the 4.0 mm opening, with a rivet covering the part of the sample protruding from the disc. Thermal contact between the sample, the disc, and a rivet that is used to cover the part of the sample protruding from the disc, is provided by Tissue Tek. The sample with the disc is rapidly frozen in nitrogen slush, mounted in the Hexland Cryotrans CT 1000 assembly, and inserted into the prechamber of the cold stage attachment where the rivet is knocked off. From that point on, the regular procedure recommended by Hexland is followed.

Initial paper received March 15, 1988
Manuscript received June 24, 1988
Direct inquiries to M. Kalab
Telephone number: 613-995-3722 x7707

KEY WORDS: Cold-stage scanning electron microscopy, Cryo-SEM, Hexland Cryotrans CT 1000 assembly, Sample holders, Tubular sample holders, Viscous foods.

Introduction

Examination of biological samples by scanning electron microscopy performed at low temperature (cryo-SEM) [10, 15, 16] is convenient for several reasons. One of them is that no chemical fixation is required as the sample is physically fixed by rapid freezing and, therefore, artefacts associated with chemical fixation are avoided. This is particularly important with foods which contain ingredients such as fat or gelatinized starch that are difficult or impossible to fix [18]. Another reason is the rapidity of specimen processing. Cryo-SEM allows for the sample to be frozen, inserted into the cryo-attachment, fractured, etched if necessary, coated with metal, and examined in the microscope within 15 min from the time the specimen is obtained [2].

Some samples are easier to prepare for examination than others. Whole small plant organs can serve as examples of superb preservation of structures by cryo-fixation [3, 4]. Microorganisms present in foods, such as fungi in mould-ripened cheeses and on surface-ripened salami, can also be preserved without developing artefacts provided that appropriate precautions are taken. Such precautions involve handling the samples in a high-humidity chamber before freezing, rapid freezing, and sputter-coating inside the cold-stage attachment in short bursts rather than in a single long interval [1]. Difficulties may be encountered with high-moisture foods (>65% moisture) such as cheese curd, soybean curd (tofu) [9], yoghurt, and cream which are more susceptible to the development of ice crystals in the aqueous phase during freezing [13] than low-moisture products (<65% moisture), such as cream cheese and other cheeses [21] or whipped cream [22]. With regard to biological samples, Moor [17] concluded that the lower the content of free water in living cells, the narrower the critical temperature zone in which crystallization of ice may take place. Apparently, this principle may also be applied to foods. Cryoprotective agents such as glycerol or dimethyl sulfoxide reduce the interval that exists between the freezing point and the recrystallization temperature and diminish the risk of ice crystal development in the sample during freezing, but incorporation of these agents into the food samples under study alters the original structure of the foods.

Ice crystal formation is related to the rate of freezing [17], which, in turn, is affected by the dimensions of the samples. The larger the sample,

the lower its freezing rate. Thus, the reduction in the size of the sample is one way of reducing the development of artefacts due to ice crystal formation [19, 20]. In practice, the size of the sample depends, to a great extent, on the sample holder used [8].

The objective of this paper is to describe the design of a brass disc compatible with the Hexland Cryotrans CT-1000 assembly which accommodates small sample holders and to describe some sample preparation aids. The accessories can be employed when using the Hexland system for food samples. without any need to modify the original equipment and with only a slight modification of the procedure used.

Materials and Methods

Sample holder for high-moisture foods

Sets of two-piece open-ended tubes made from sterling silver are used as holders for viscous foods such as cream and stirred yoghurt. The lower tube is longer (2.7 mm) than the upper tube (1.2 mm). The outer diameter of both tubes is 1.2 mm and the wall thickness is 0.15 mm. A brass block has been designed to accommodate 3 samples (Fig. 1) and to fit the standard specimen holder of the Hexland Cryotrans CT-1000 assembly (Fig. 2) (Hexland Electron Microscopy Division, Oxford Instruments Ltd., Eynsham, Oxford OX8 1TL, UK). In order to achieve compatibility, the brass block (Figs. 1 and 3) has the same dimensions (3.7 mm high and 10 mm in diameter) as the original 10-mm diameter aluminum disc supplied with the attachment. The characteristic feature of the block is three openings, each 1.2 mm in diameter and 2.7 mm deep, to accommodate the silver tubes. Small orifices (0.6 mm in diameter) are drilled through the bottoms of the openings to facilitate mounting of the sample-filled tubular holders and for cleaning. Shafts containing setscrews have been drilled in at a 122° angle to the radial direction. This design allows for longer shafts, which provide a better anchoring system for the setscrews than the shorter shafts that would result from their drilling in the radial direction. The setscrew shafts are accessible through grooves machined in the brass block. In addition, the block has a central hole into which a handle can be screwed. The handle is used to manipulate the block during sample loading (Fig. 4) and also to insert the loaded block into the Hexland Cryotrans CT 1000 assembly (Fig. 5).

In preparation for the cold-stage work, the handle is screwed into the brass block and together with a pair of insulated tweezers and the screwdriver, which will be used to tighten the setscrews, pre-cooled by immersion in liquid nitrogen.

A sample of the viscous food destined for examination by cryo-SEM is placed in the bottom silver tube which is held with a pair of forceps. The tube may be filled with the sample using a Pasteur pipet (which has been drawn out into a fine tip) until the sample protrudes at one end. Using a second pair of forceps, the shorter tube is placed over the protruding sample. This operation may also be performed using only one pair of special forceps designed by Sleytr and Umrath [22]. The filled tubes, held together in a vertical position by the sample, are individually frozen in Freon 12 cooled to its freezing point of -150°C with liquid nitrogen and transferred into liquid nitrogen in a shallow insulated container. There, the tubes are mounted into the pre-cooled brass block using the pre-cooled tools. Each pair of tubes is pushed down into one of the three holes in the block with the longer tube inserted first so that the junction in the coupled tubes is flush with the top of the block and the short tube projects above. The tubes are then fixed in place by tightening the setscrews while the brass block is held by its handle. The brass block is then inserted into the Hexland specimen carrier assembly (Fig. 5), which had been pre-cooled to -196°C, and is secured with a setscrew. The handle is then removed from the block and the Hexland assembly is lifted out of liquid nitrogen and inserted into the pre-chamber of the attachment.

Fracturing is effected by using the back edge (not the blade) of the cooled scalpel to knock off the top silver tube from the bottom one. After examining the fracture face in the microscope and etching it if required, the sample may be withdrawn into the pre-chamber, sputter-coated with gold, returned into the microscope, and photographed.

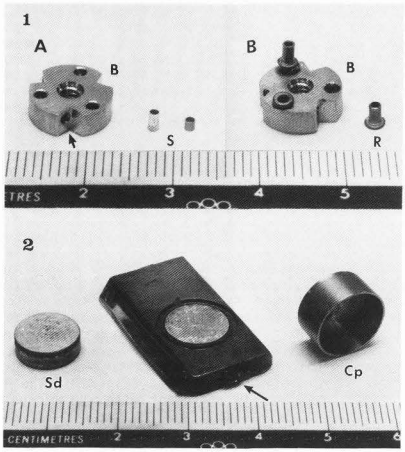
A similar brass block accommodating slightly wider (outer diameter, 1.5 mm, inner diameter, 1.1 mm) brass rivet-type holders of the same length as the silver holders and open at both ends but having a lip (rim) at one end, has also been made and tested (Fig. 1B). It may be used to examine food samples by cryo-SEM without fracturing. The sample is loaded into the rivet allowing the convex meniscus to protrude onto the lip. Without covering it with another rivet, the sample is rapidly frozen by plunging the rivets individually into Freon 12 cooled to -150°C as described above. All steps from here are the same as described earlier but the fracturing step is omitted.

Sample holder for low-moisture foods

The original Hexland design of a central blind-ended rivet sunken into the aluminum disc has been replaced with a more robust open-ended rivet (inner diameter of 4.0 mm, wall thickness of 1.0 mm), also sunken centrally in the disc. The bottom orifice is temporarily covered with a disc of sticky tape cut to size and is removed after the completion of cryo-SEM for cleaning the holder.

For semi-solid low-moisture food samples such as mayonnaise, whipped cream, or thick salad dressings, the bottom orifice of the sunken rivet is covered and the aluminum disc secured to the specimen carrier of the Hexland assembly. The sample is loaded into the rivet in the aluminum disc with a spatula until the sample bulges slightly above the top of the rivet. Another rivet is placed on top and filled with the sample (Fig. 6). The whole specimen carrier, including the sample, is then plunged into nitrogen slush in accordance with the instructions by Hexland, inserted into the pre-chamber of the attachment where fracturing is effected by knocking the top rivet off from the bottom one as described above. The procedure from here on follows the manufacturer's instructions.

For solid low-moisture food samples such as cheese, the same double-rivet set-up is used, again with the bottom orifice covered with a sticky tape. A drop of Tissue Tek (Hexland) is placed in the bottom rivet and the other rivet is placed on its



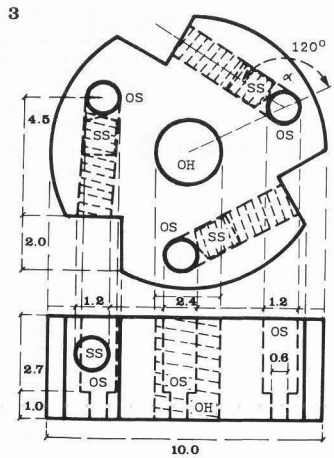


Fig. 1. Brass blocks (B) with concentric holes for three 2-piece silver tubes (S) with an outer diameter of 1.1 mm (Fig. 1A), or open-ended rivets (R in Fig. 1B) with an outer diameter of 1.5 mm, used as sample holders. The central hole in the brass block accommodates the block handle which facilitates tightening of the setscrews (arrow). Numbers on the ruler indicate centimeters.

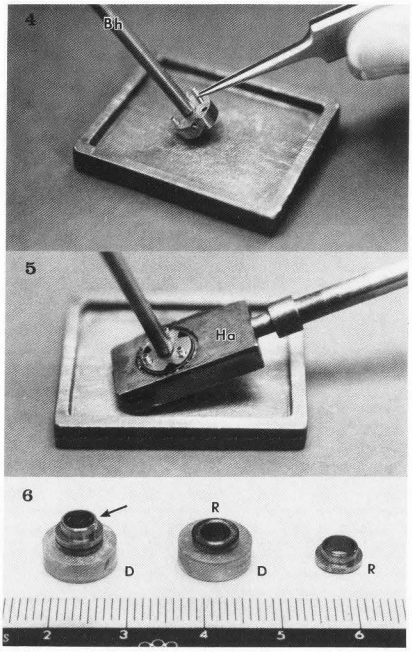


Fig. 2. Hexland standard aluminum sample disc (Sd) used as a holder in the Cryosystem CT 1000 assembly. After insertion, the disc is tightened using a setscrew (arrow). Cap (Cp) is used to cover samples that are sensitive to moisture loss [1]. Numbers on the ruler indicate centimeters.

Fig. 3. Diagram of the brass block. Dimensions are shown in millimeters. OS = openings for sample holders, OH = opening for block handle, SS = setscrews (the shafts have been drilled at an angle α of 122° from the radial direction).

Fig. 4. Mounting of the silver tubes with the sample frozen inside into the brass block. The brass block is manipulated by its handle (Bh). In this demonstration, liquid nitrogen was not used in order to obtain clear photographs of the block.

Fig. 5. Insertion of the brass block with the samples in place into the Hexland assembly (Ha).

Fig. 6. A rivet (R, 4.0 mm inner diameter) sunken in a hole drilled in the original aluminum disc (D) is used as a holder for low-moisture samples. Another rivet (arrow) is placed on top of the sunken rivet.

top. The solid food is sampled using a #1 cork borer (inner diameter of 4.0 mm) with the plunger raised. Then, using the plunger, the sample is extruded into the rivets, frozen in nitrogen slush, and fractured as described above.

Results

High-moisture food samples

The reduction in size of the samples held in small tubular silver holders makes it possible to freeze viscous food samples, such as cream (Fig. 7), more rapidly than in larger holders and to obtain good micrographs by using cryo-SEM. It is easier to freeze the samples individually in cooled Freon than in nitrogen slush. Yet, despite the small size of the sample, ice crystals occasionally develop even when precautions are taken. There are either no ice crystals in the area adjacent to the silver tube or, if they are present, they are small. Larger ice crystals may be found at the centre of the sample. This indicates that the freezing rates are different in different areas of the sample. Usually there are, however, some areas unaffected by the crystal growth which are suitable for photography.

Hexland makes available a wide range of specimen holder blocks (discs) including locking and non-locking types, to accommodate different types of samples placed in rivet-like holders. With regard to the locking holders, the manufacturer employs two methods for securing the rivets to the holder, either with a screw set into the top of the disc or with a pivoting ring which anchors 3 rivets at the same time. With the modified design based on a similar type of holder described earlier [12], loading of the samples into the brass block and manipulating the block is made easy by using a handle designed to be temporarily screwed into the block and removed afterwards. In contrast, using the commercially available holders, the manipulation of the samples and the adjustment of the ring to lock or free the rivets under liquid nitrogen may not be as easy. The rivets in the original specimen holder are placed farther away from the thermocouples than the tubes in the design described here. In addition, using the original holder, it is difficult to fracture all three samples by knocking off the top rivets from the bottom ones using the scalpel. The difficulty is caused by the central pin, which is part of the mechanism that allows the locking of the rivets; it stands in the way of the scalpel and is permanently attached there. Only two fractures can be effected easily even when three samples are mounted in the disc. The lack of a special tool to be used for pivoting the commercially available discs under liquid nitrogen constituted another reason for developing an easier way to accomplish that goal.

It was found that freeze-fracturing may not always be required to show the microstructure of the sample. It is possible to freeze-etch samples, such as stirred yoghurt, thus allowing examination of a large area of the superficial layer of the frozen sample which is free from any ice crystals [7], because it was in direct contact with the coolant and thus frozen more rapidly than the interior of the sample. A yoghurt sample that was allowed to protrude slightly from the single brass rivet (1.5 mm in diameter) was used. The protruding surface, in direct contact with the cryogen upon freezing, had negligible ice crystal development at the sample surface. Casein micelle chains and clusters seen in the sample (Fig. 8) are similar to those seen in yoghurt by conventional SEM [14], i.e., examination at ambient temperature of chemically fixed samples that had been dried. Cryo-SEM thus provides useful confirmation of the reliability of conventional methods.

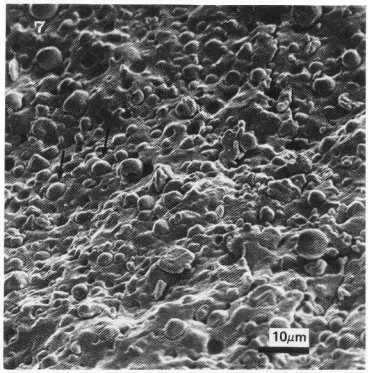
Low-moisture food samples

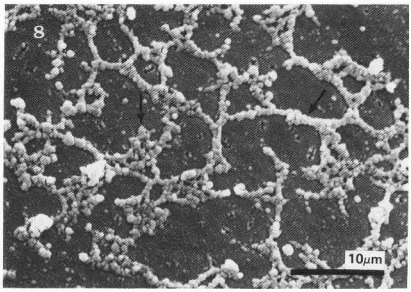
The reason for using the more robust (4.0 mm in diameter) sample holders for low-moisture foods is a higher resistance of low-moisture foods to ice crystal formation during freezing. Thus, the freezing rate is not as critical as with high-moisture foods. The larger holders are easier to handle and fill with the sample than the narrow silver tubes. Fig. 9 shows a commercial salad dressing frozen in the holder. There is no ice crystal damage to the structure of the sample, as is apparent from the appearance of the aqueous phase in which fat globules and other ingredients are dispersed. Starch is noticeable in another salad dressing sample that had been extensively freeze-etched (Fig. 10). The image of the starch is in agreement with the micrographs of starch in cooked pasta [11].

Discussion

Many of the specimen holders available for cold-stage work today, including those available from Hexland, are patterned on the basic design by Echlin and Burgess [6]. These authors have demonstrated a support for 4 samples in rivet-type holders made from silver, but these holders were not individually secured. The use of "fine-bore" silver tubes, 1 mm in diameter, was suggested earlier [12] for freeze-fracturing followed by replication with platinum and carbon in a Polaron freeze-fracturing module. Silver has high thermal conductivity and even thin-walled tubes made from this metal have sufficient mechanical strength [19]. Open-ended tubular holders are easy to fill with liquid samples whereas air bubbles are usually trapped in "blind" holders. By accommodating 3 holders in the block (similar to the design put forth by Echlin and Burgess [6], as well as the design of the Hexland holders), time is spent efficiently on the instruments and the chances of obtaining artefact-free zones are increased compared to working with only one sample per run. This is important particularly with high-moisture food samples, which are susceptible to ice crystal damage.

Even with the refinements suggested, the study of high-moisture food products is not easy. Problems may develop when freeze-etching is carried out. The aqueous phase in most milk products, particularly freshly coagulated curd and yoghurt, contains varying concentrations of solutes such as lactose, mineral salts, and whey proteins. These substances appear as a fine efflorescence on the freeze-etched surface after pure water is removed by sublimation of the ice. In such cases, where an insight into the protein matrix is required and where possible, cold stage SEM should be complemented by SEM carried out at ambient temperature and using chemically fixed samples which had been dehydrated, freeze-fractured, and critical point dried [5, 13]. In other cases,





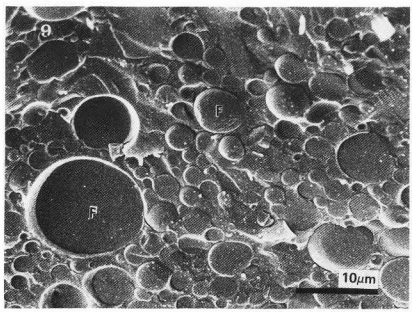


Fig. 7. Cryo-SEM of a whipping cream sample (35% fat) individually frozen in tubular silver holders in Freon 12 at -150°C. After fracturing at -150°C, the temperature was gradually increased to -83°C for freeze-etching at $1x10^{-5}$torr; 15 min after fracturing, the temperature was decreased to -150°C and the sample was coated with gold and examined at 10 kV. Freeze-etching exposed fat globules (arrows).

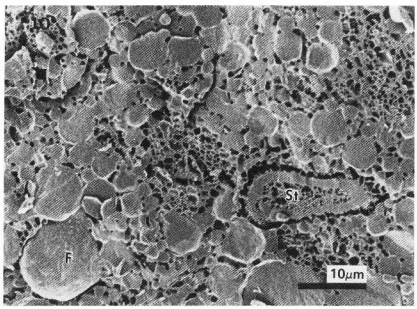


Fig. 8. A stirred yoghurt sample placed in a brass rivet, 1.5 mm in diameter, was examined by cryo-SEM without fracturing following freeze-etching of the superficial layer. Casein particle chains and clusters (arrows) are in agreement with the microstructure of yoghurt as seen by conventional SEM [14].

Fig. 9. Cryo-SEM of a commercial salad dressing placed in the single rivet holder reveals numerous fat globules (F) of various dimensions.

Fig. 10. Cryo-SEM of another salad dressing sample shows fat globules (F) and starch (St).

e.g., low-moisture foods, an examination of the freshly freeze-fractured but unetched surface may provide the required information.

Acknowledgements

The authors thank Mr. Al Bingham, SEMOptics, Ltd., Nepean, Ontario, Canada, for assistance with the design and manufacture of the brass blocks, the holders, and the handle. Appreciation is expressed to Dr. G. H. Haggis for useful suggestions. Electron Microscope Unit, Plant Research Centre, Agriculture Canada in Ottawa provided facilities. Contribution 765 from the Food Research Centre and 1117 from the Plant Research Centre.

References

1. Allan-Wojtas P, Yang AF. (1987). Solutions to difficulties encountered in low-temperature SEM of some frozen hydrated specimens: Examination of *Penicillium nalgiovense* cultures. J. Electron Microsc. Tech. 6, 325-333.
2. Beckett A, Read ND. (1986). Low-temperature scanning electron microscopy. In: Ultrastructure Techniques for Microorganisms. HC Aldrich, WJ Todd (eds.), Plenum Press, New York, NY, 45-86.

3. Brookes B, Small E. (1988). Enhanced floral analysis by low temperature scanning electron microscopy. Scanning Microsc. 2(1), 247-256.
4. Cavdek V, Lott JNA, Kerr P. (1987). Cell wall wrinkling and solute leakage in imbibing squash and carrot seeds. Scanning Microsc. 1(3), 1413-1421.
5. Cross RHM, Hodgson AN. (1986). The role of SEM cryo techniques in inter-microscopic correlation. Proc. XIth Int. Cong. Electron Microsc., Kyoto, Japan. Jap. Soc. Electron Microsc., Tokyo, Japan, 2229-2230.
6. Echlin P, Burgess A. (1977). Cryofracturing and low-temperature scanning electron microscopy of plant material. Scanning Electron Microsc. 1977;I:491-500.
7. Echlin, P. (1973). Scanning microscopy at low temperature. In: Freeze-Etching Techniques and Applications. EL Benedetti, P Favard (eds.), Soc. Franc. Microsc. Electr., Paris, 211-222.
8. Fuchs W, Fuchs H. (1980). The use of frozen-hydrated bulk specimens for X-ray microanalysis. Scanning Electron Microsc. 1980;II: 371-382, 296.
9. Gallant DJ, Saio K, Ogura K. (1976). The observation of high-moisture foods with a scanning electron microscope using the cryotechnique. JEOL News 14(2), 6-10.
10. Gilkey JC, Staehelin LA. (1986). Advances in ultrarapid freezing for the preservation of cellular structure. J. Electron Microsc. Tech. 3(2), 177-210.
11. Kaláb M. (1983). Electron microscopy of foods. In: Physical Properties of Foods. M Peleg, EB Bagley (eds.), AVI Publ. Co., Inc., Westport, CT, 43-104.
12. Kaláb M. (1981). Electron microscopy of milk products: A review of techniques. Scanning Electron Microsc. 1981;III:453-472.
13. Kaláb M. (1984). Artefacts in conventional scanning electron microscopy of some milk products. Food Microstruc. 3, 95-111.
14. Kaláb M, Allan-Wojtas P, Phipps-Todd BE. (1983). Development of microstructure in yoghurt - A review. Food Microstruc. 2, 51-66.
15. Lott JNA, Carson J, Kerr P. (1985). Cryogenic preparation of samples for scanning electron microscopy. Microsc. Soc. Can. Bull. 13(3), 5-9.
16. Lott JNA, Kerr P. (1986). Use of cryogenically prepared samples in the scanning electron microscopic study of dry-to-wet transitions. Scanning Electron Microsc. 1986;III:979-986.
17. Moor H. (1973). Cryotechnology for the structural analysis of biological material. In: Freeze-Etching Techniques and Applications. EL Benedetti, P Favard (eds.), Soc. Franc. Microsc. Electr., Paris, 11-19.
18. Plattner H, Bachmann L. (1982). Cryofixation: A tool in biological ultrastructural research. Int. Review Cytol., Vol. 79, GH Bourne, JF Danielli (eds.), Acad. Press, Inc., New York, NY, 237-304.
19. Robards AW, Sleytr UB. (1985). Low Temperature Methods in Biological Electron Microscopy. Elsevier Science Publishers B. V. (Biomedical Division), Amsterdam, the Netherlands, 324-326.
20. Schmidt DG, Henstra S, Thiel F. (1979). A simple low-temperature technique for SEM of cheese. Mikroskopie (Vienna) 35, 50-55.
21. Schmidt DG, Van Hooydonk ACM. (1980). A scanning electron microscopical investigation of the whipping of cream. Scanning Electron Microsc. 1980;III:653-658, 644.
22. Sleytr UB, Umrath W. (1974). A simple fracturing device for obtaining complementary replicas of freeze-fractured and freeze-etched suspensions and tissue fragments. J. Microsc. 101(2), 177-186.

Discussion with Reviewers

J. A. Sargent: It would be helpful to give the address of the supplier of the sterling silver tubes.
Authors: The tubes were obtained from local jewellers' supplies and findings stores. In Canada, they may be ordered from Imperial Smelting and Refining Co., Ltd., 451 Denison, Markham, Ontario.

J. A. Sargent: Is there not a possibility of altering the structure of the sample through shearing effects at the neck of a drawn pipette?
Authors: The only samples which are handled with a drawn pipette are the ones which are liquid, *e.g.*, milk or cream. These samples consist basically of particulate matter suspended in a liquid. The small diameter of the tip of the drawn pipette (approx. 0.5 mm in diameter) is considerably larger than the particles in suspension and so shearing effects in this kind of high-moisture samples should not be a problem.

J. N. A. Lott: The filling of small diameter tubes without getting air bubbles is often a problem. The discussion could probably be extended as to how this was done.
Authors: The fact that the tubes are open-ended, we believe, is the most important single factor which allows the samples to be prepared without the inclusion of air bubbles. As a precaution, the first bit exiting from the drawn pipette, which may contain bubbles, is blotted away and only the sample which is bubble-free is used to fill the tubes. In addition, the tubes are filled to overflowing and only then is the top tube added.

D. J. Gallant: In my experience, samples are not always completely preserved even by rapid freezing. I observed, *e.g.*, that potato cells which did not show ice crystals immediately after freezing, suddenly developed large ice crystals when the temperature of the Hexland Cryotrans assembly was increased to -85°C. This finding has been reproducible. Have you observed such a phenomenon and could you outline the temperature variations which occurred with the various steps of your experiments and which may explain the drying effect observed with the swollen starch granule in Fig. 10?
Authors: Yes, we have observed this phenomenon also, *e.g.*, with celery and parsnip (but not with processed foods such as yoghurt) following the increase of their temperature to -85°C. The temperature profile of the vegetables and the processed foods was identical: the samples were frozen in nitrogen slush, inserted onto the cold stage at about -190°C, and maintained at -160°C except for freeze-etching which was carried out at -85°C.