A New and Rapid Method for the Electron Microscopic Examination of Fats

G. G. JEWELL and M. L. MEARA,
British Food Manufacturing Industries Research Association, Randalls Road, Leatherhead, Surrey, England

Abstract

A replica technique was developed to study the crystal structure of fats using the electron microscope. The fats were frozen prior to replicating, and it was shown that differing rates of freezing produce structural differences within the fat. The use of Teepol solutions permitted a degree of separation of the solid and liquid phases, and the differences between crystals of the solid phase were shown.

Introduction

Our present knowledge of the crystalline structure of fats has been based on the light microscopic studies carried out by such workers as Malkin (1), Bailey (2) and Hoerr (3), and on the x-ray diffraction studies of, for example, Lutton (4), Malkin (5), Hoerr (6) and Chapman (7). However these two techniques yield results which do not cover the entire range of possible crystal sizes, inasmuch as the light microscope will enable measurements to be made on crystals down to a value in the region of 1 μ, while x-ray investigation is capable of giving information on unit cells and repeating units measured in nanometers.

Using the electron microscope, with its resolution limit of about 0.5 nm, it should be possible to produce direct visual observations of crystals whose sizes lie in precisely the range (1 nm to > 0.1 μ) which neither light microscopy nor x-ray techniques can span.

The routine methods for specimen preparation for electron microscopy can be classified into two main groups: (a) techniques based on cutting very thin sections 30–80 nm thick, and (b) techniques based on an examination of the surface topography, by preparing a surface impression or replica.

The thin sectioning technique has been applied by Wortmann (8) to fatty materials such as butter, but the procedure adopted required the specimen to be treated with osmium tetroxide solution for a period of six months. In view of this time period it was concluded that this technique would be of little use for a fairly rapid assessment method, whereas the application of a replica technique should overcome this most undesirable delay.

A replica is normally prepared by the vacuum deposition of a layer of carbon onto the surface of the specimen. To enhance the differences in surface topography, a contrasting agent, usually a heavy metal such as platinum, is vacuum deposited on to the carbon layer from a precisely known angle, this procedure being known as shadowing. The substrate is then removed from the replica either by stripping or solvation, and the replica is examined in the electron microscope. Since the angle of application of shadowing material is known, and the length of the shadow so produced may be observed and subsequently measured in the electron microscope, the height of the original feature which gave rise to the shadow can be determined by the application of trigonometry.

Bradley (9) had described a number of modifications applicable to the replication procedure, which have been developed for the examination of a wide variety of biological and nonbiological materials. However, it was very quickly appreciated from our preliminary experiments, that the high temperatures produced by the vacuum sputtering of carbon and platinum onto the surface of fatty material produced severe degradation of the sample by localized melting. It was considered that freezing the sample prior to replication might well remove the problem of melting. The present paper describes the successful modification of the replica technique whereby it is now possible to provide a reliable and fairly rapid technique for the examination of fatty materials.

Materials and Methods

The descriptions of the techniques developed, and the results obtained, are restricted to those from commercially supplied samples of lard and an all vegetable oil shortening, although, to date, over 50 different fats and blends have been examined in these laboratories.

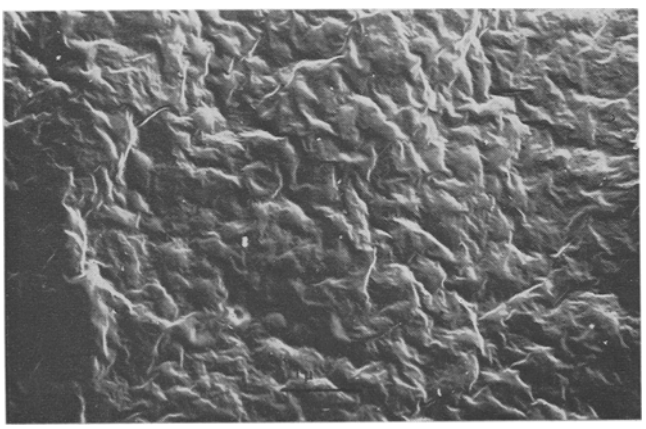

FIG. 1. A replica of a rapidly cooled shortening. Note the absence of crystalline features. (Reduced approximately 45%)

FIG. 2. A replica of a rapidly cooled lard. Crystals (C) Can be seen beneath a masking layer of amorphous material. (Reduced approximately 45%)

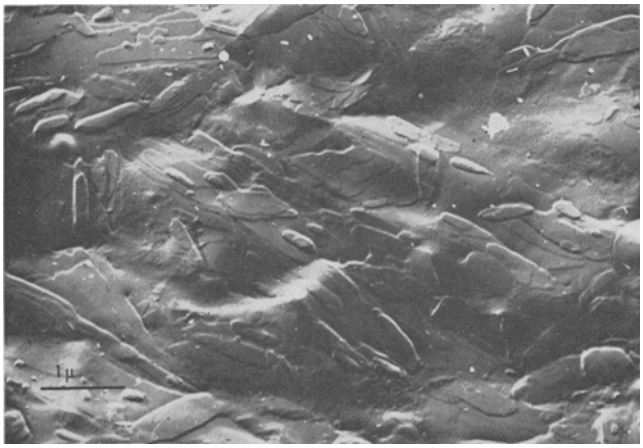

FIG. 3. A replica of a slowly cooled shortening. Numerous small crystals can be seen. (Reduced approximately 45%)

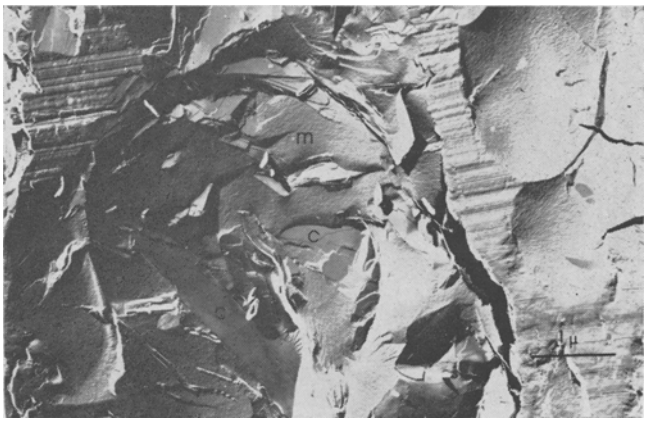

FIG. 5. A replica of a freeze fractured shortening. Crystals (C) are embedded in an amorphous matrix (M). (Reduced approximately 45%)

The fats had been tempered commercially, and upon receipt were held at 20 C prior to the application of the replication procedure. Although the composition of the fats was not determined, the solid fat index was assayed by a standard dilatometric procedure and gave the following values at 20 C: lard 23.3, and vegetable oil shortening 21.3.

All treatments prior to replication were carried out on a thin smear of fat supported on a ¼ in. square × 0.005 in. thick molybdenum foil. The effect of two different chilling rates was investigated: (a) a very rapid rate of cooling produced by plunging the sample into liquid freon which had been pre-cooled to −150 C in liquid nitrogen, and (b) a much slower rate of cooling, produced by placing the sample on a brass block (mass 200 g) which had been pre-cooled to −150 C.

For reasons which will be discussed later, some of the samples were exposed to the action of diluted detergent solution for a period of 11 hr prior to cooling. The strength of the detergent solution used was dependent on the fat under examination, but generally a 10–35% aqueous solution of Teepol proved to be satisfactory, the actual concentration being determined on the basis of the strongest solution which did not cause the fat to float off the molybdenum foil, or otherwise disperse. The sample which had been washed in detergent was rinsed in distilled water to remove Teepol solution and the excess water was removed by allowing it to drain onto a filter paper. The final traces of water were removed by pumping under a vacuum of the order of 0.5 mm Hg. The samples which had been detergent-washed were then cooled by either of the two methods previously described. The chilled samples were placed in the vacuum coating unit, onto brass blocks which had been pre-cooled to −150 C. It has been shown by Comer and Turley (10) that reversing the order of deposition of carbon and platinum respectively, leads to a greater degree of resolution. Therefore, when a vacuum of the order of 1×10^{-5} torr was attained, a thin layer of platinum 5–10 nm thick was sputtered onto the fat surface at a shadowing angle of 11° to the horizontal. A thicker layer of carbon (100–200 nm) was then evaporated onto the top of the platinum, from a source normal to the fat surface. The samples were then removed from the coating unit, and the carbon-platinum replicas were released from the fat surface by the following solvent extraction procedure. The samples were dipped first into methanol which removed the frost from the still very cold sample. The bulk of the fat was dissolved by immersion in chloroform for 15 min; the replicas were then transferred to electron microscope support grids, these then being rinsed with ether to remove the last traces of fat.

To clarify certain problems which arose from the interpretation of the data obtained from the experiments carried out using different cooling rates, preliminary experiments were also carried out using a freeze-fracture technique. In this technique the samples were rapidly frozen in liquid freon, and transferred to a vacuum coating–freeze fracture unit.

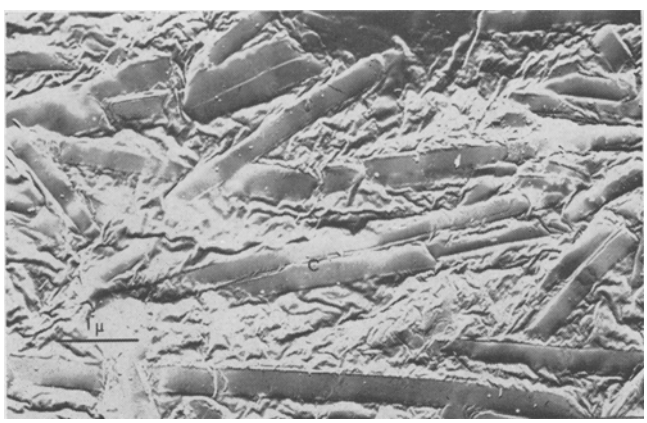

FIG. 4. A replica of a slowly cooled lard. Well defined crystals (C) can be seen. (Reduced approximately 45%)

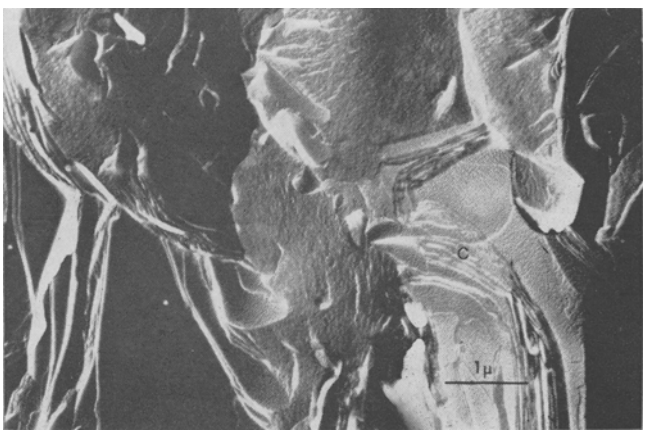

FIG. 6. A replica of a freeze fractured lard. A well defined crystal edge (C) is visible and areas of the crystal exhibit a pimple like appearance. (Reduced approximately 45%)

When a high vacuum was attained the sample was fractured by means of a microtome blade. The fractured surface thereby exposed was replicated in the manner described above. This technique makes possible the examination of a freshly cleaved frozen surface as opposed to an original surface which had been frozen.

All replicas were examined on a Hitachi HS-7S electron microscope operating at 50 kv.

Results

The photographs have been produced from an intermediate negative, hence the shadows produced by differences in surface topography will appear black.

An examination of the replica from a sample of shortening which had been rapidly cooled in liquid freon (Fig. 1) revealed that virtually no crystalline material was present in the surface of the sample. A replica from lard prepared under the same conditions (Fig. 2) indicated that the surface of the sample was composed of amorphous material but there was evidence for the presence of large crystals lying beneath this masking amorphous layer.

Samples which had been more slowly cooled prior to replication differed from the rapidly cooled specimens by showing a much greater degree of crystallinity. Thus the shortening sample (Fig. 3) showed the presence of numerous crystals, whose sizes were considerably smaller than those crystals observed in the lard sample (Fig. 4).

Samples which had been rapidly frozen and then freeze-fractured (Fig. 5,6) showed surfaces which consisted of large areas of relatively amorphous material, but also regions containing well defined crystalline material. Again, the crystals present in the shortening were smaller than those observed for lard.

When the shortening had been treated with a 15% Teepol solution, the appearance of the surfaces produced by either rapid or slow cooling were virtually identical (Fig. 7,8), both revealing a highly crystalline surface, with crystals of a variety of shapes, whose sizes were in the range 250–1000 nm. Treatment of the lard sample with 15% Teepol gave a pattern indicating the presence of crystals virtually identical with those described for Figures 2 and 4. However, when the concentration of the Teepol solution was increased to 35%, either rapid or slow rates of chilling produced the type of surface shown in Figures 9 and 10. Thus both rates of chilling produced surfaces which contained well defined crystals of sizes up to 8 $\mu \times 1$ μ. It was also observed that the lard crystals are composed of layers of crystallites, each of these crystallites being of the order of 4–6 nm thick. Furthermore some of the lard crystals contained areas which exhibited a regular pimple like structure (Fig. 6,9) each pimple being of the order of 6–8 nm in diameter.

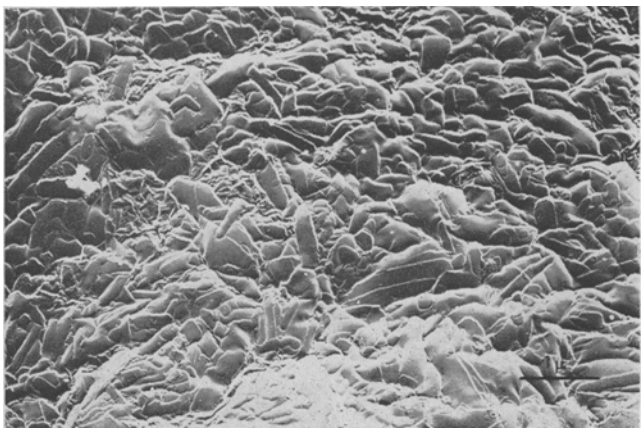

FIG. 8. A replica of a vegetable oil shortening which had been treated with 15% aqueous Teepol, then slowly cooled. A highly crystalline surface is evident. (Reduced approximately 45%)

Discussion

It is well known that the fats under investigation will, at ambient temperature, consist of a mixture of solid and liquid phases, the ratio of solid to liquid fat depending on the temperature. It is suggested therefore that the observed difference in surface structure produced by the different rates of cooling may be connected with differences in the fate of the original liquid phase. A very rapid cooling might be expected to convert the liquid into a noncrystalline, vitreous, or amorphous phase, whereas slower cooling might well induce a certain amount of crystallinity. If this is so, then since the same pattern of a vitreous or amorphous phase is obtained in the case of rapidly chilled material, it must be deduced that the surface layer of the fats investigated must at ambient temperatures have consisted of a liquid film. The more ordered pattern associated with the somewhat slower

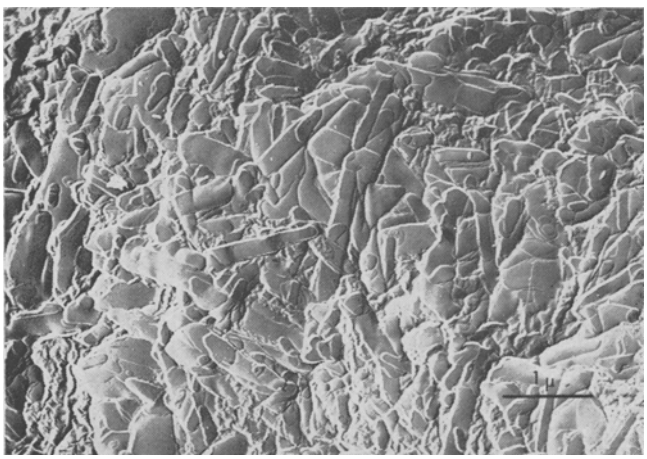

FIG. 7. A replica of a vegetable oil shortening which had been treated with 15% aqueous Teepol, then rapidly cooled. A highly crystalline surface is evident. (Reduced approximately 45%)

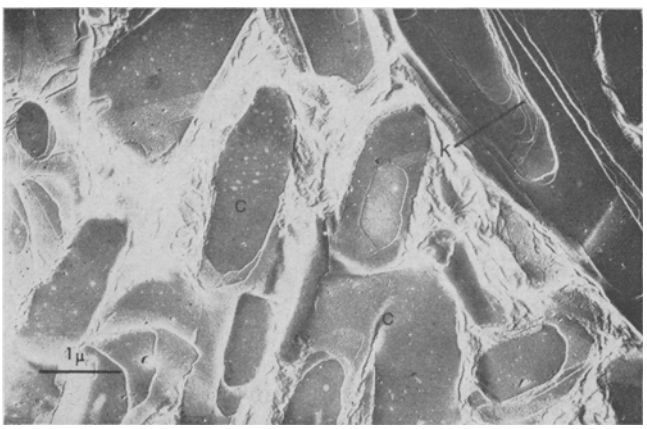

FIG. 9. A replica of a lard which had been treated with 35% aqueous Teepol, then rapidly cooled. Well defined crystals (C) are visible, each crystal being composed of a stack of crystallites (K). (Reduced approximately 45%)

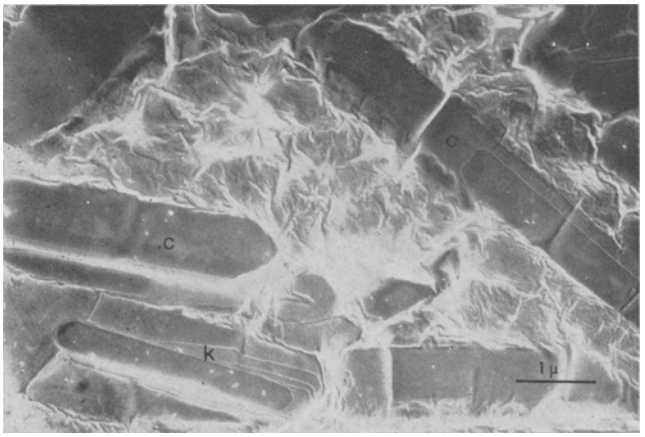

FIG. 10. A replica of a lard which had been treated with 35% aqueous Teepol, then slowly cooled. Crystals (C) composed of layers of crystallites (K) can be clearly seen. (Reduced approximately 45%)

rate of cooling is taken as being due to the crystalline structure derived from the original liquid phase. In the case of the rapidly chilled lard, large crystals can be seen lying beneath the amorphous surface layer, and this is considered to be due to the original solid phase present in the lard at ambient temperature. The concept of solid fat phase being entirely surrounded by liquid phase receives support from the freeze fracture experiments since the exposed fractured surface of the rapidly cooled specimens have been shown to consist of areas of highly crystalline material surrounded by regions of amorphous or vitreous material.

The separation of the solid from the liquid phase in a fat by the action of a surface active agent is similar in principle to the now commercially available processes, based on the Henkel patents (British 724,222), whereby fatty acids can be separated from a mixture of solid and liquid fatty acids. Our experience of this has shown that solid glycerides can be separated from liquid glycerides relatively easily using the smear technique, or at least a solid surface can be prepared for further investigation as described in the experimental section. An examination of the pattern obtained from any one of these fractions, prepared either by the rapid or slow cooling techniques show that they are virtually identical and must therefore represent the inherent solid phase in the fat sample taken.

It is of considerable interest to note that the inherent solid phases of the different fats examined differed considerably in crystal sizes. Other results indicate that the size of large crystals, as determined by the electron microscope investigation, correspond well with those derived from light microscopy. The demonstration that lard crystals are comprised of layers of thickness 4–6 nm is also of interest since this value is in the order of the x-ray long spacings for the solid components of lard. The possible significance of the pimple structure observed on some of the lard crystals will be discussed in a later paper.

Since the time taken to prepare and examine the replica, excluding the time taken for Teepol washing, where necessary, is of the order of 2 hr, it is considered that this technique can be utilized as a fairly rapid method for the examination of fat crystals. The differences in crystal habit exhibited by a wide variety of fats are now under investigation in these laboratories and relationships between these parameters and the performance characteristics of the fats are being sought.

ACKNOWLEDGMENTS

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