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GRAIN STRUCTURE AND END-USE PROPERTIES

Y. Pomeranz

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Abstract

Practical implications of grain structure relate to every step from grain development and production through marketing to processing, utilization, and consumption. The structure and adherence of the hulls may contribute to protection of grain during germination or malting and protection against insect infestations. Germ retention during threshing and separation during processing depend on the germ structure and location in the kernel. The subaleurone and central endosperm layers differ in cell size, shape, and structure and in composition, especially with regard to protein contents and quality. The main factors in grain hardness are the intrinsic hardness of the main components, the strength of interaction within the cell, and the interaction of individual cells to produce overall grain structure.

Endosperm structure and hardness is related to wheat conditioning, to breakage in milling, and to the structure and composition of the milled flour particles. Milling quality is governed by morphological characteristics of the wheat kernel and its mechanophysical properties and by the methods of grinding and separation. Reducing changes in texture and structure during drying of maize and rice are important in minimizing breakage during handling, storage, and transportation, dust formation, and infestation. Differences in grain structure are expressed in differences in composition, gradients of components in grain tissues, and end-use properties. Those differences have important nutritional implications. New microscopic methods to determine grain structure, composition, and end-use properties have the potential of contributing to improved nutritional quality and utilization of cereals by modifying-restructuring grain morphology through classical plant breeding and genetic engineering.

Introduction

Grain structure is an expression of grain composition as it reflects properties from the standpoint of plant physiology. The plant does not synthesize or incorporate components into structures unless they have a specific function in preservation or propagation of the species. Cereal chemists and technologists, on the other hand, are interested in another set of properties --the function the grain or its fractions can perform in the production of nutritious foods, that have good shelf life, and are acceptable to the consumer.

Thus, in a way, grain structure forms the link between composition that is the source of our basic knowledge of biological systems and utilization of those components in food production. For optimum utilization of cereal grains, knowledge of their structures and compositions is required. The practical implications of kernel structure are numerous. They relate to the numerous steps of grain production, harvest, storage, marketing, and utilization. Some of the implications are listed in Table 1. Discussed here, in detail, are primarily studies that deal with wheat and barley. Other cereal grains are reviewed briefly.

Kernel Structure - General

The cereal grain is a one-seeded fruit, called a caryopsis, in which the fruit coat is adherent to the seed. As the fruit ripens, the pericarp (fruit wall) becomes firmly attached to the wall of the seed proper. The pericarp, seed coat, nucellus, and aleurone cells form the bran. The embryo occupies only a small part of the seed. The bulk of the seed is taken up by the endosperm, which constitutes a food reservoir.

The floral envelopes (modified leaves known as lemma and palea), or chaffy parts, within which the caryopsis develops, persist to maturity in the grass family (MacMasters, 1962). If the chaffy structures envelope the caryopsis so closely that they remain attached to it when the grain is threshed (as in rice and most varieties of oats and barley), the grain is considered to be covered. However, if the caryopsis readily separates from the floral envelopes on threshing,
Table 1. Some Implications of Kernel Structure

<table>
<thead>
<tr>
<th>Significance in</th>
<th>Parameter</th>
<th>Effect</th>
<th>Commodity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshing</td>
<td>Germ damage or skinning</td>
<td>Reduced germinability, impaired storability</td>
<td>All cereal grains</td>
</tr>
<tr>
<td>Drying</td>
<td>Cracks, fissures, and breakage; hardening</td>
<td>Reduced commercial value; lowered grade, impaired storability, dust formation, reduced starch yield</td>
<td>Mainly corn and rice</td>
</tr>
<tr>
<td></td>
<td>Discoloration</td>
<td>Reduced commercial value, lowered grade</td>
<td>Mainly rice</td>
</tr>
<tr>
<td>Marketing</td>
<td>Breakage</td>
<td>Reduced commercial value in food processing</td>
<td>Mainly corn and rice</td>
</tr>
<tr>
<td>General use</td>
<td>High husk: caryopsis ratio or high pericarp: endosperm ratio</td>
<td>Reduced nutritional value—as food or feed</td>
<td>All cereal grains</td>
</tr>
<tr>
<td>General use</td>
<td>Kernel shape and dimensions, proportions of tissues in the kernel, distribution of nutrients in the tissues</td>
<td>Yield of food products; nutritional value of cereal (or cereal products) as food or feed</td>
<td>All cereal grains</td>
</tr>
<tr>
<td>Malting</td>
<td>Germ damage, skinning, or inadequate husk adherence</td>
<td>Reduced germinability, uneven malting</td>
<td>Mainly barley</td>
</tr>
<tr>
<td>Milling</td>
<td>Uneven surface, deep crease or uneven aleurone</td>
<td>Reduced milling yield</td>
<td>Mainly wheat and rice</td>
</tr>
<tr>
<td>Milling</td>
<td>Steely texture</td>
<td>Increased power requirements, starch damage, high water absorption, difficulty in airclassification</td>
<td>Wheat and malt milling</td>
</tr>
<tr>
<td>Germination-Malting</td>
<td>Starch granule size</td>
<td>Uneven degradation</td>
<td>All cereal grains</td>
</tr>
<tr>
<td>Consumption-Nutrition</td>
<td>Distribution and composition of protein</td>
<td>Change in nutritional value</td>
<td>All cereal grains</td>
</tr>
</tbody>
</table>

as with common wheats, rye, hull-less barley, and the common varieties of corn, the grain is considered to be naked.

The structure of the wheat kernel is shown in Fig. 1. The dorsal side of the wheat grain is rounded, while the ventral side has a deep groove or crease along the entire longitudinal axis. At the apex or small end (stigmatic end) of the grain is a cluster of short, fine hairs known as brush hairs. The pericarp, or dry fruit coat, consists of four layers: the epidermis, hypodermis, cross cells, and tube cells. The remaining tissues of the grain are the inner bran (seed coat and nucellar tissue), endosperm, and embryo (germ). The aleurone layer consists of large rectangular, heavy-walled, cells. Botanically, the aleurone is the outer layer of the endosperm, but as it tends to remain attached to the outer coats during wheat milling, it is considered by millers as the innermost bran layer.

The embryonic axis consists of the plumule and radicle, which are connected by the mesocotyl. The scutellum serves as an organ for food storage. The outer layer of the scutellum, the epithelium, may function either as a secretory or as an absorption organ. In a well-filled wheat kernel, the germ comprises about 2-3% of the kernel, the bran 13-17%, and the starchy endosperm the remainder. The inner bran layer (the aleurone) is high in protein, fat, and minerals, whereas, the outer bran layers (pericarp, seed coats and nucellus) are high in cellulose, hemicelluloses, and minerals. The germ is high in proteins, lipids, sugars (chiefly sucrose), and minerals;
the starchy endosperm consists largely of starch grains surrounded by protein.

Grains of other cereals are similar in structure to wheat. The corn grain is the largest of all cereals. The kernel is flattened, wedge-shaped, and broader at the apex than at its attachment to the cob. The aleurone cells contain much protein and oil and also contain the pigments that make certain varieties appear blue, black, or purple. Two types of starchy endosperms—horny and floury—are found beneath the aleurone layer (Masters, 1962). The horny endosperm is harder and contains a higher level of protein. In dent corn varieties, the horny endosperm is found on the sides and back of the kernel and bulges in toward the center at the sides. The floury endosperm fills the crown (upper part) of the kernel, extends downward to surround the germ, and shrinks as corn matures.

In a typical dent corn, the pericarp comprises 6%, the germ 11%, and the endosperm 83% of the kernel. Flint corn varieties contain more horny than floury endosperm.

The common varieties of oats have the fruit (caryopsis) enveloped by a hull composed of certain floral envelopes. In light thin oats, hulls may comprise as much as 45% of the grain; in very heavy or plump oats, they may represent only 20%. The hull normally makes up <30% of the grain.

Rice is a covered cereal; in the threshed grain (or rough rice), the kernel is enclosed in a tough siliceous hull, which renders it unsuitable for human consumption. When this hull is removed during milling, the kernel (or caryopsis), comprised of the pericarp (outer bran) and the seed proper (inner bran, endosperm, and germ), is known as brown rice or sometimes as unpolished rice. Brown rice is in little demand as a food. Unless stored under very favorable conditions, it tends to become rancid and is more subject to insect infestation than the various forms of milled white rice. When brown rice is subjected to further milling processes, the bran and germ are removed and the purified endosperms are marketed as white rice or polished rice.

Hull and Bran Layers

The structure and adherence of the hull are important in protecting the germinating grain and in the malting process. One reason that barley is uniquely suited for malting is that a cementing layer is present between the hull and the caryopsis. The hull restricts excessive seedling growth without adversely affecting the desirable enzymic degradation of insoluble high molecular weight materials. The adhering hull also protects the seedling from mechanical damage during turning of the malt, and provides a filtration bed during the extraction of soluble malt components in the mashing process.

The hull, as such or as a result of the high concentration of silica, can slow the attack of storage insects on rice and barley. The palea and lemma in barley are held together by two hook-like structures (Fig. 2 a & b). In rice, the ability of these structures to hold the palea and lemma together without gaps is probably variety dependent. Varieties of rice that had many gaps and separations had greater insect infestations than did varieties with tight husks.

Apparently the bran (pericarp, seed coats, nucellus and aleurone) affords little protection against insect infestations, because more insects consistently develop in brown rice than in either rough rice or milled rice. Lack of resistance to infestation is probably due to the thinness of the bran, which allows easy insect penetration, and to the large quantities of lipid and protein present in the aleurone which provide nourishment to the insects.

The outer pericarp layers of wheat (epidermis and hypodermis) have no intercellular spaces and are composed of closely adhering, thick-walled cells. The inner layers of the pericarp, on the other hand, consist of thinner-walled cells and often contain intercellular spaces, through which water can move rapidly and in which molds are commonly found. Similarly, molds can enter through the large intercellular spaces at the base of the kernel where the grain was detached from the plant at harvest and where there
is no protective epidermis. An intact grain stores much better than a damaged or ground grain; deteriorative changes (i.e., rancidity, off-flavors, etc.) occur slowly in the whole grain but quite rapidly in ground grain. The hull, apparently, prevents rancidity by protecting the bran layers from mechanical damage during harvesting and subsequent handling. Once rough rice is dehulled, it rapidly becomes rancid, primarily because of the oxidation of free fatty acids released by the action of lipase. The lipids and lipase are normally compartmentalized in the aleurone and germ cells. Cell disruption may cause mixing of the cellular constituents. Possibly, the dehulling process and subsequent handling of dehulled rice disrupt the aleurone cells and allow the rice to become rancid. The hull, therefore, appears to be necessary to prevent cell disruption during harvesting, storage, and handling.

The Germ

The site of the germ in the kernel and the extent to which the germ is protected by adjacent layers determine whether it will be retained intact during threshing and, thus, the usefulness of the grain for seeding or malting. The ease with which the germ is removed from the caryopsis during milling depends on several factors. The germ is a separate structure and generally can be easily separated from the rest of the cereal grain. However, the scutellar epithelium (located next to the endosperm) has fingerlike cells, which in wheat are attached to one another for about one-third of their length. The free ends protrude toward the adjacent starchy endosperm cells. The protruding epithelial cells may secrete an amorphous cementing material between germ and endosperm. If some of this material projects into the spaces between the fingerlike cells of the scutellar epithelium and into the folds of the scutellar structure, it may be difficult to separate the germ from the endosperm unless the cementing material is softened. The softening may be accomplished by steeping, as in corn wet milling, or by conditioning, as in wheat milling (MacMasters, 1962). In rice, a layer of crushed cells separating the scutellar epithelium from the starchy endosperm provides a line of easy fracture; hence the germ can be removed intact with minimum effort.

Germ separation is also facilitated by the fact that the germ takes up water faster and swells more readily than the endosperm. The strains resulting from differential swelling contribute to easy separation in milling.

The Subaleurone Layer

The uniqueness of the subaleurone layer in wheat was studied intensively by Kent and co-workers. The subaleurone endosperm in wheat consists of a region of distinctive starchy endosperm cells, one or more layers deep, adjacent to the aleurone cells on the outside and to the inner endosperm on the inside. In hard wheats, the subaleurone layer forms a fairly complete shell around the inner endosperm (except in the regions of the scutellar epithelium and the base of the crease). In soft wheats, the shell is often discontinuous and at the points of discontinuity endosperm cells with typical inner endosperm characteristics extend out to the aleurone layer (Kent, 1966). The two types of endosperm (subaleurone and inner) differ in cell size and shape, size and abundance of starch granules, and proportion of protein. Subaleurone endosperm cells are generally small and cubical; those of the inner endosperm are larger and either needle-shaped (prismatic) of polyhedral (central endosperm).

The ratio of gluten to water soluble proteins is higher in the subaleurone than in the central endosperm layer (Simmonds, 1971). The distribution of starch granules also shows marked differences in different areas of the endosperm. In the subaleurone cells, the starch granules are intermediate in size with relatively few small or secondary granules. In contrast, the mid-endosperm cells are packed with large primary and small secondary granules with the storage protein forming a thin matrix between them.

The differences in structure and composition of various starchy endosperm layers have significant implications in milling. Stock from the first break rolls consists of a coarse fraction, semolina, derived primarily from the center of
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the endosperm (Simmonds, 1971, 1972). On further grinding this yields, especially from high protein wheats, a first reduction flour of high starch content. Subsequent milling of the overlattings from the first break removes flour endosperm cells progressively closer to the aleurone layer, and the final break rolls yield a product of high protein content. The reason is the high protein content of the subaleurone layer. Subaleurone and inner endosperm in the coarse (over 35 μm) air-classified fractions of flour milled from hard red winter wheat had average protein contents of about 45 and 11%, respectively. Cells of subaleurone endosperm were reduced to particles below 35 μm size less readily than were cells of inner endosperm. Consequently, subaleurone endosperm cells concentrated in the coarse air-classified fractions and raised the protein content of the latter (Kent, 1966).

Kernel Hardness

Over the years, few subjects have been more controversial and enigmatic than the biochemistry of wheat hardness. The major factors involved in wheat hardness are the intrinsic hardness of the main components (starch and protein), the strength of the interaction with the cell, and the interaction of individual cells to produce the overall grain structure (Stenvert and Kingswood, 1977).

All immature wheat grains are vitreous; as maturation proceeds, some grains remain vitreous, while others become mealy. Endosperm cells in soft wheat contain starch granules embedded in a friable matrix which is readily crushed by the rollers during milling, releasing the starch granules cleanly and with little damage. The endosperm cells of hard wheats, on the other hand, tend to shatter rather than powder, breakage of both starch granules and protein matrix occurring. Thus, the degree of starch damage and the amount of protein matrix carried at the surface of individual kernels is higher in hard than in soft wheats (Simmonds, 1971). According to Kent (1969), proteins are largely responsible for hindering the disruption of the endosperm cell contents during roller milling and hard wheat endosperm would make difficult separating the starch granules from the protein matrix—as is characteristic in hard wheats. A discontinuous matrix structure would allow the ready release of starch granules as found with soft wheats.

Stenvert and Kingswood (1977) studied the influence of a range of factors on wheat hardness with particular reference to the physical structure of the endosperm protein matrix. Differences in hardness involved the continuity of the protein matrix and the strength with which it physically entrapped starch granules. The primary determinant of wheat hardness is genetically controlled and relates to factors that influence compactness of endosperm cell components.

Relation between physical structure and some chemical constituents and milling performance of bread and feed wheats were studied by Nierle and Elbayy (1978). Vitreous kernels of varieties Jubilar and Caribo were higher in protein content, sedimentation value, and wet gluten than mealy grains. The ratio of soluble protein content to total protein content and of gliadin to glutenin in vitreous kernels was lower than in mealy grains. Electrophoretic and gel chromatographic studies suggested higher hydrogen bonding forces between protein molecules of vitreous than of mealy kernels. Non starch lipids were more extractable in mealy kernels, presumably, because of the less compact nature of the protein matrix. Mealy grains produced higher amounts of break flour with lower mineral content than vitreous kernels. It is questioned whether the reported differences are related to kernel
structure or to associated variations in protein contents and distribution.

**Cellular Structure and Endosperm Breakage**

Endosperm structure and hardness is related to breakage in milling and to the structure and composition of milled flour particles. Microscopy has demonstrated fundamental differences between the cellular structure of soft and hard wheats and flour particles obtained from such wheats (Greer and Hinton, 1950; Greer et al., 1951; Kent and Jones, 1952). Greer et al. (1951) suggested that whereas flour particles of soft wheats consist of broken cells, those of hard wheats consist of entire cells or groups of cells, each including its individual cell wall. Kent and Jones (1952) presented a method of characterizing a flour in terms of its cellular structure. The proportions of flour particles were classified according to 1) the part of the wheat endosperm from which they are derived (peripheral, prismatic, or central), 2) the number of cell units comprising the particle, 3) the relative intactness of the cells, and the relative extent of cell wall covering, depending on the type of wheat (hard or soft), part of the milling system (break or reduction), and conditioning.

Schultze and MacMasters (1962) showed that endosperm breakage occurs across cell walls, not between the walls of adjacent cells. The cell-wall particles consistently were portions of walls of two adjacent cells, with the middle lamella which cements them. Breakage of the cell walls was invariably transverse, rather than along the middle lamella.

Moss et al. (1980) examined six wheat cultivars and their mill brans with the SEM. Endosperm removal (flour) from bran was related to the cleavage pattern of the grains. Good bran cleanup and high flour yield were associated with inter-cellular cleavage. When fracture took place through the contents of the endosperm cells more endosperm adhered to the bran. The hardness of the endosperm cells determined the nature of the cleavage pattern. In a hard wheat, a continuous protein matrix is formed around all the cell contents resulting in the boundary between the cell wall and cell contents becoming a zone of weakness. Hence cleavage was intercellular. The cell contents could then act as a single entity and the whole cell could be removed from the bran by shear forces imparted during milling. Some forces were also redirected towards the bran, fragmenting it into small pieces. In a soft wheat, air spaces and discontinuities in the protein matrix made the cell fragile and the shear forces were not redirected, but passed through the cell rather than removing it cleanly from the bran. Increasing the water content of the wheat prior to milling favored intra-cellular cleavage. Thus, it was concluded, that the relationship between bran clean-up and bran fracturing can be optimized by balancing intrinsic hardness and grain moisture content; this is the basis of wheat conditioning.

**Wheat Conditioning**

Water absorption during wheat conditioning largely governs its milling behavior (Bradbury, et al., 1960). Moss (1973) studied varietal differences in grain morphology as they affect water penetration into the wheat, overall conditioning, and the milling process. It has been suggested that varietal differences are affected by thickness and composition of the outer cuticle and testa, the extent to which the outer epidermal and inner parenchymal cells have been compressed, and the number and size of protein masses in the subaleurone endosperm cells (Hinton, 1955).

According to Drews (1979), an important factor in conditioning and separation of the starchy endosperm is the amount of water absorbing and swelling components. The amount is highest in bran, lower in the aleurone, and lowest in the starchy endosperm. Attack of grain by microorganisms may degrade the cellulosic and semi-cellulosic materials in the outer layers and reduce their water binding and swelling capacities. Sprouting can have profound effects on degradation of those materials and significantly affect their milling properties. The presence of relatively large amounts of pentosans in the starchy endosperm of rye and triticale may significantly influence their milling properties. According to Meuser and Metz (1979), starch is the main wheat component that can be relatively easily modified in its physicochemical properties by milling. This is accomplished by varying the degree of starch damage and results in changes in water binding capacity and susceptibility to a-amylase degradation. Such damage is small in soft wheat because conventional milling separates easily the starch granules from the protein matrix. An increase in water binding capacity that accompanies increased severity of milling soft wheats, is due to an increase in the number of particles rather than to an increase in starch damage (Meuser and Klingler, 1979). Those findings are especially relevant in light of the studies by Evers and Lindley (1977) on particle size distribution of starch starch in the endosperm of 12 wheats. Granules below 10 μm diameter accounted for about 1/3 to 1/2 of the total weight or endosperm starch. As the samples were representative of wheat, it is suggested that small granules may be of great importance in determining flour and dough properties. In a study of the size distribution of starch granules in endosperm of different sized kernels of the wheat cultivar Maris Huntsman, the number of starch granules was greater in large-plump than in small-plump or shrivelled kernels (Brocklehurst and Evers, 1977). In all three kernel types more than 1/3 of the total starch weight was contributed by granules less than 10 μm diameter (B type granules). The proportion of small granules was significantly greater in large-plump kernels than in the other two types.

Studies by Butcher and Stenvert (1973) determined by an autoradiographic technique that the rate of water penetration into the kernel differed among Australian wheat cultivars. Moss (1977) used an improved technique, applicable directly to whole sections. This enabled a more precise location of the conditioning water to be made than could be previously achieved using halves of labeled wheat grains. Within 1 hr the labeled water penetrated into the aleurone cells and in

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In many cases into the starchy endosperm to a depth of 50 to 60 μm. The embryo and scutellum also absorbed the water with great rapidity. Subsequent penetration into the starchy endosperm was delayed for several hours. The cells of the embryo and scutellum appeared to bind the water more strongly than the aleurone and after 48 hr rest time were still more heavily labeled than the other components of the wheat grain (Moss, 1977).

In a recent publication, based on the above findings, the relation between optimum conditioning, moisture level, and grain hardness was reported (Anon., 1977) (Table 2).

Table 2. Relation Between Wheat Type, Grain Hardness and Optimum Conditioning Moisture

<table>
<thead>
<tr>
<th>Wheat Type</th>
<th>Grain Hardness (PSI)</th>
<th>Optimum Conditioning Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. Hard</td>
<td>9-13</td>
<td>16.5-17.5</td>
</tr>
<tr>
<td>Hard</td>
<td>14-19</td>
<td>15.5-16.5</td>
</tr>
<tr>
<td>Intermediate</td>
<td>20-25</td>
<td>14.5-15.5</td>
</tr>
<tr>
<td>Soft</td>
<td>25-30</td>
<td>13.5-14.5</td>
</tr>
</tbody>
</table>

Factors which affect the rest time after sampling and before milling include: (a) initial moisture content, (b) protein content, and (c) grain hardiness. An increase in moisture content from 8 to 12% can reduce rest time three-fold due to a reduction in the water binding potential of grain components at higher initial moisture contents, thus allowing a more rapid movement of water. An increase in wheat protein content decreases the rate of water penetration. This is due to protein retarding the moisture movement because of its water binding capacity and because it contributes to a more ordered endosperm structure. The latter relates to the role of grain hardness in slowing moisture movement (Anon., 1977).

Milling Score-Flour Yield

Milling quality is governed by morphological characteristics of the wheat kernel and its mechano-physical properties and by the methods of grinding and separation (Meuser and Klingler, 1979). The size and shape of the crease affect yield and composition of flour because the bran in the crease area is difficult to separate from the starchy endosperm (Fig. 3 a & b). The volume occupied by the crease has been calculated to range from 0.7 to 1.9% of the total grain volume and its relative size affects the milling process in terms of ability to extract flour and its potential for providing a hospice for fungal growth at grain maturity. Some interest is being shown in breeding grain without a crease (Kingswood, 1975). The flour yield from such grain would be greatly increased. Kosina (1979) determined, in a study of several European wheats, gross morphological differences in kernel structure that are likely to affect flour yield. The coefficients of variation were largest for the height of the endosperm cavity and lowest for the thickness of the aleurone layer (Table 3).

Table 3. Coefficients of Variation of Gross Morphological Parameters of the Wheat Kernel

<table>
<thead>
<tr>
<th>Morphological Parameter</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosperm cavity, height</td>
<td>314</td>
</tr>
<tr>
<td>Endosperm cavity appendices, thickness</td>
<td>44</td>
</tr>
<tr>
<td>Subaleurone layer, thickness</td>
<td>33</td>
</tr>
<tr>
<td>Crease width</td>
<td>27</td>
</tr>
<tr>
<td>Crease depth</td>
<td>18</td>
</tr>
<tr>
<td>Aleurone layer, thickness</td>
<td>12</td>
</tr>
</tbody>
</table>

It would, thus, seem that in breeding types of wheat that produce the highest yield in terms of wheat flour, changing the aleurone layer thickness is not a highly promising approach. According to MacMasters (1962), the milling engineer is troubled by the constant minor variations in the thickness of the aleurone layer. The irregular thickness of the aleurone layer makes it difficult to scrape all of the starchy endosperm from it, in milling. That the starchy endosperm and the aleurone layer are part of one tissue, rather than separate, merely adherent.
wheats of poor milling quality corresponded to a wheats. which accumulate abnormally in wheats that are difficult to mill. Seeborg and Barmore suggested that the increased yield of that material from wheats of poor milling quality corresponded to a greater amount of endosperm cell walls in such wheats. 

Marked expansion in recent years of the area planted to high-yielding wheat varieties not suitable for baking (i.e. Maris-Huntsman in the United Kingdom and Clement in the Netherlands) required development of a simple and quick testing procedure to distinguish between baking and feed wheats (Bolling and Meyer, 1975). The authors emphasized the need to distinguish clearly between inferior milling and inferior baking properties. Milling and baking properties may be related, but not necessarily. 

Histological studies of Bolling and Meyer (1975) have shown that in acceptable cultivars, a fairly strong adhesion between the aleurone-pericarp layers facilitated their separation from the starchy endosperm. In inferior wheats, the subaleurone layer is rich in protein, adheres strongly to the aleurone layer, and cannot be separated efficiently. In some inferior cultivars, the porous structure of the pericarp facilitates shattering and increases the bran content of flour (Bolling and Meyer, 1975).

Meuser and Reiner (1977) studied the possibility of influencing milling and, indirectly, baking properties. The authors investigated the effects of particle size and structure of flour particles and their composition in a wheat milled under various conditions. This was followed by determining the influence of raw material quality on the structure of flour particles from various hard wheats and the possibility of modifying processing characteristics of flours from a single wheat by altering milling parameters. 

Wheat hardness is a varietal characteristic that governs particle size of milled products (Meuser and Klingler, 1979). Generally, soft wheats produce "smooth" and hard wheats "gritty" flours. The inherent capacity of hard wheat to produce semolina or farina may be important. The range of particle sizes in milling hard wheats is wider than in milling soft wheats. The former also are more granular than the latter. The strong adherence between protein and starch in hard wheats results also in greater homogeneity in composition of the milled particles than in soft wheat flours. The extensive mechanical degradation of soft wheats in milling increases their water binding capacity to such a large extent, that their baking properties may be impaired. Consequently, it may be desirable to conduct the milling process in such a manner that most particles are in the 40 to 90 μm range. This modification of the milling process and resultant changes in particle size distribution could not counteract however, the inferior performance of feed wheats (Meuser and Klingler, 1979).

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Yamazaki and Donelson (1972) found a high negative correlation for white layer cake volume vs. mass-median diameter of laboratory-milled cake flours obtained from pure-variety wheats. Cake volume was also associated inversely with mass median diameters of straight-grade and coarsely milled flours and directly with the quantity of sifted meal from wheats milled to obtain patent flours for cake baking. Varietal differences in cake potential for those wheats appeared to be associated largely with inherent differences in endosperm friability. 

Predicting Milling Behavior

The problem of predicting the milling behavior of wheat by the use of simple chemical laboratory tests has occupied cereal chemists for many years. 

The endosperm cell walls of seven Pacific Northwest (PNW) wheat cultivars ranging from excellent to poor in milling quality, were studied by Wolf et al. (1952) to determine differences in content and composition of water-insoluble hemi-celluloses. In all varieties, degradation of endosperm cell walls in transsections treated with 1% H2SO4 or 1% KOH was greatest near the aleurone and decreased toward the crease. The degradation was greatest in the first two or three cell layers just beneath the aleurone layer. The cell walls were degraded over a greater area in varieties of excellent milling quality than in those of poor milling quality, but varieties of intermediate milling quality behaved erratically (Wolf et al., 1952).

Larkin et al. (1952) found with PNW white wheats that the thickness of cell walls in the endosperm near the aleurone layer was least in the wheats of best milling quality. Differences between varieties, however, were smaller than differences between different parts of the grain. The relationship was independent of year and location (MacMasters et al., 1957). Similar results were reported for soft wheats by Popham et al. (1961) but in that case the year of growth had a significant effect on milling quality. In the PNW, for HRS wheats, the correlation coefficient between the pentosan value and milling score was -0.74 and -0.84 for selected winter and spring wheat, respectively. The correlation coefficients between pentosan and flour yield, bran weight, and bran cleanup were significant at the 1% level (Weswig et al., 1963).

Medcalf et al. (1968) reported that durum pentosans contained a higher proportion of arabino-bose, and thus more branching, than hard red spring (HRS) pentosans. Similarly, durum pentosans were somewhat higher in molecular weight than HRS pentosans. The differences were significant but relatively small. It was postulated, however, that a small difference in the degree of branching might alter markedly the degree and type of interaction of polysaccharides with proteins. Differences in molecular weight also could alter the interaction mechanism and water sorption. These differences could account, in part, for the differences in endosperm properties between durum and HRS wheats.

Mares and Stone (1973a) showed that endosperm flours had an ultrastructure similar to...
primary cell walls, having a microfibrillar phase embedded in an amorphous matrix. Chemical studies showed that the walls were largely composed of polysaccharide and that some protein was also present. The predominant polysaccharides are arabinoxylans of which one-third are soluble in water, the remainder requiring alkaline reagents for solubilization. The authors isolated, fractionated, and characterized what endosperm cell walls, free from non-endospermic cell walls, in flours from three wheats. The isolated cell walls were similar in proportions of the polymeric components and the monosaccharide composition of the walls and the wall fractions. The appearance of endosperm cell walls in situ and in wall isolates was examined by light microscopy, scanning and transmission electron microscopy. SEM showed apparent moulding of the walls on the cell contents and different fracture patterns of prismatic and central cells. The cell walls have a microfibrillar skeleton embedded in the amorphous matrix components.

In a subsequent study (Mares and Stone, 1972b), it was found that the endosperm cell walls are composed mainly of arabinoxylan and of some cellulose, glucomannan and protein. The postulated presence of cellulose is of particular interest. Stenvert and Moss (1974) applied a detergent extraction technique to quantitatively separate the outer layers of the wheat grain from the starchy endosperm. Milling studies demonstrated that the extraction technique allowed an accurate estimate of the flour yielding potential provided that the milling system was capable of fully realizing the flour milling capacity. This is much easier said than done. In certain instances the true flour yielding potential of a wheat could not be judged by the detergent method due to differences in optimum conditioning requirements, ease of separation of bran from the endosperm, dressing properties and sensitivity to moisture level. Grain hardness and test weight of hard and soft wheats were not related significantly to flour yield.

Those studies showed similarities in the chemistry of the cell walls of wheats of diverse milling quality and seem to suggest that neither variations in proportions of the component polysaccharide fractions nor in their composition are responsible for meaningful differences in milling properties. The possibility to develop a simple chemical test that relates cell wall material to varietal differences in milling cannot be excluded. It is not likely, however, that such a single test could account for differences in cell wall thickness, content, distribution, composition, and many other factors which govern milling quality. And, last but not least, we have no standardized or optimized acceptable milling test that could be used as a reference basis.

**Grain Drying**

Changes in texture and structure during drying of corn and rice are important in minimizing breakage during handling. Excessive cracks reduce value of corn for producing foods such as breakfast cereals. Harsh heat treatment during grain drying may reduce starch yields, impair quality of the starch, and create difficulties in corn wet-milling. If used for alcoholic beverages, overheated corn may also cause difficulties in beer brewing and in distillation. The starch granules are embedded in a proteinaceous matrix that hardens during overheating. The hardened matrix protects the starch from enzyme attack and conversion to alcohol. Broken corn is more easily attacked by insects and produces more grain dust than whole grain and creates many problems in handling, transportation, and storage. In rice milling, harsh drying and accompanying structural cracks substantially reduce yields of head rice and increase amounts of "broken," and thus cause economic losses to the miller. The method of rice drying may also affect the texture and color of the milled rice and result in off-color, especially objectionable browning.

**Nutritional Implications**

The chemical composition of different cereal grains varies widely, since it is influenced by genetic, soil, cultural and climatic factors. Amounts of proteins, lipids, carbohydrates, pigments, vitamins and total ash vary; mineral elements present also vary widely. Cereals are characterized by relatively low protein and high carbohydrate contents; the carbohydrates consist essentially of starch (90% or more), pentosans, and sugars.

The various components are not uniformly distributed in the kernel. The hulls and bran are high in cellulose, pentosans and ash; the germ is high in lipid and rich in proteins, sugars, and generally, ash. The endosperm, which contains the starch, has a lower protein content than the germ and the bran (in some cereals), and is low in fat and ash.

Furthermore, the various proteins are not distributed uniformly in the kernel. Thus, the proteins fractionated from the inner endosperm of wheat consist chiefly of approximately equal amounts of prolamins (gliadins) and glutelins (glutenins). The embryo proteins consist of nucleoproteins, albumin (leucosin), globulins, and proteoses; in wheat bran prolamins predominate with smaller quantities of albumins and globulins.

Breeding efforts to improve the nutritive value of cereal grains have concentrated on increasing protein content without decreasing protein quality (mainly retaining lysine concentration in the protein). The significance of protein distribution in the endosperm depends on the type of product that is likely to be consumed. In the production of highly refined milled products, in which some of the subaleurone layer is removed, a high concentration of protein in the subaleurone layer would not be desirable. However, if the whole kernel is to be consumed, distribution of protein in the kernel is of limited nutritional consequence.

In all cereal grains, the storage protein forms a matrix which surrounds the starch granules. Protein body initiation and formation of the matrix protein were studied recently by Bechtel et al. (1982a, b). Those studies suggested a role for the Golgi apparatus in the
initiation of protein bodies. The Golgi apparatus in wheat may function as a concentration organelle to establish foci for accumulation of proteins. Protein bodies that formed in the cytoplasm were transported to the central vacuoles where the protein body membrane and tonoplast fused and deposited the protein granules into the vacuole. Protein granules in the vacuole enlarged by three mechanisms: 1. addition of membranous vesicular material of various types, 2. addition of flocculent material, and 3. fusion of granules with other newly deposited protein granules. Fig. 4 shows the relationships among kernel dry weight, water content and protein per kernel. The dry weight per kernel increased consistently during development whereas the moisture content remained relatively constant during the first 12 days and then dropped rapidly. Protein content per kernel increased consistently during caryopsis development and closely paralleled the dry weight data.

Fusion of protein granules to form a large protein mass is illustrated in Fig. 5 and Fig. 6. This method of protein enlargement increased noticeably during 14 to 17 days after flowering and peaked at 21 to 28 days after flowering. It is of interest that wheats harvested at about 21 to 28 days after flowering (or about 10 preripe) have maximum loaf volume potentialities. There is good indirect evidence that up to a certain level, increased protein body fusion is concomitant with increases in loaf volume potential.

The concentration of protein increases from the inner to the outer starchy endosperm. The increase may be relatively gradual, as in some soft wheats (Kent 1966), or quite steep, as in some high-protein wheat types in which some of the outer subaleurone cells contain few, if any, starch granules. Since the subaleurone region in rice is only several layers thick and lies directly below the aleurone, the subaleurone layer can be easily removed during milling. It is, therefore, desirable either to mill rice as lightly as possible for a consumer acceptable product, or to breed cultivars with an increased subaleurone layer, or cultivars with a more even distribution of protein throughout the endosperm.

Sullins and Rooney (1974) used SEM to illustrate differences in corn endosperm structure that account for differences in nutritive value of the grain. High-lysine corn has a reduced amount of protein bodies in the endosperm. SEM of soft endosperms for normal, opaque-2 or modified opaque-2 corn showed loosely packed, nearly round starch granules associated with thin sheets of protein and many intergranular air spaces (Robutti et al., 1974). The hard endosperms had tightly packed, polygonal starch granules associated with a continuous protein matrix, and no intergranular air spaces. Normal hard endosperms had zein bodies embedded in the protein matrix; modified hard endosperms did not. Starch damage was greater in the hard endosperm than in the soft.
because of a stronger adhesion between starch and protein. The low density and opaqueness of soft endosperm were attributed to the intergranular air spaces. Interaction between protein matrix and starch granules during drying explains the shape of starch granules.

Seckinger and Wolf (1973) studied the structure of grain sorghum endosperm protein of commercial hybrids and experimental lines with the transmission electron microscope (TEM) and SEM. Vitreous endosperm showed a well developed, two-component structure consisting of concentric-ringed protein bodies (2-3 μm in diameter) embedded in an amorphous matrix protein. On the basis of solubility properties of the proteins, they suggested that the protein bodies were the site of prolamin (kafferin) deposition and that the matrix protein was the site of glutelination. Distribution of protein within the sorghum grain was similar to that within other cereal grains in that the peripheral vitreous area of the kernel had the highest protein content. Interior areas had gradually decreasing amounts of protein. The waxy sorghum varieties contained fewer spherical protein bodies and, therefore, were essentially round and covered with a thin sheet of protein. Embedded in the protein sheet were relatively large spherical protein bodies. The hard or translucent endosperm portion was characterized by a tightly packed structure with no air spaces. The starch granules were polygonal and covered with a thick protein matrix. Embedded in the protein matrix were protein bodies. Sullins and Rooney (1973) conducted light microscopy (LM) and SEM studies of the peripheral endosperm of waxy and nonwaxy endosperm sorghum varieties. Sorghum varieties are known to differ widely in endosperm type (i.e., yellow, sugary, waxy, and nonwaxy). In feeding trials sorghum varieties are known to differ extensively in feed efficiencies than nonwaxy varieties. Sullins and Rooney (1973) indicate that the modification of the endosperm in germinated barley commences at the dorsal (nonfurrowed) surface of the grain. Palmer found that the rate of endosperm modification depended more on the effective dispersal of hydrolytic enzymes than on the total amounts of these enzymes in the grain. Microscopical analyses showed that starch granules and hemicellulosic materials of the cell walls were coated with proteinaceous materials. Proteases play a more active role than carbohydrases in the conversion of hard barley into friable malt.

Changes in the aleurone layer and in the starchy endosperm of steeped, malted, and kilned barley were examined by SEM (Pomeranz, 1972). The surfaces of aleurone cells in steeped barley were highly pitted. The walls of aleurone cells were progressively degraded during malting and kilning. Aleurone grains increased in diameter during steeping and were further distorted during kilning. Partial breakdown of cell walls in the center of the starchy endosperm of malted barley was accompanied by extensive dissolution of the protein matrix and the "freezing" of small starch granules that previously were embedded in that matrix; the effect on the appearance of the starch granules was small. In the central endosperm of kilned barley malt, the cell-wall dissolution was extensive and was accompanied by mechanical breakdown of the large starch granules. A study on modification in a kilned malt was completed recently in our laboratories (Fretzdorf et al., 1982) using a combination of histochemistry, light microscopy, and transmission and scanning electron microscopy. Hydrolysis of cell walls, proteins, and starch was most extensive in the starchy endosperm area adjacent to the scutellar epithelium (Figs. 7 to 10). Some hydrolysis occurred in areas adjacent to the aleurone layers; hydrolysis decreased as distance increased from the embryonic to the scutellar end. Since the aleurone layer to the center of the starchy endosperm (Fig. 11). While no rigid sequence of hydrolysis was observed, generally, cell wall hydrolysis was more extensive than protein hydrolysis.
Fig. 7. Diagramatic scheme of barley or malt showing regions where various sections (X's = cross section, L's = longitudinal section) were taken.

Hydrolysis, and starch hydrolysis seemed to take place gradually in the late stages of malting and kilning. Small starch granules were hydrolyzed more extensively than large granules.

The SEM was used to follow the modification in malting of a low-protein barley and a high-protein cultivar (Pomeranz, 1974). In the low-protein cultivar, the protein matrix degraded extensively, and some of the degraded protein was deposited in the kilned malt on large starch granules. In the high-protein cultivar, much of the protein matrix was intact and some protein was retained in the form of a modified but coherent and continuous thick film covering the starch granules. It was suggested that the thick film is responsible for difficulties in malting high-protein barleys, for reduction of wort extract, and for persistence of undegraded proteins, which enhance chill haze formation in beer.

Palmer (1974) suggested on the basis of SEM that during malting hydrolytic enzymes migrate into the endosperm to disrupt and solubilize mainly the cell walls, complex protein materials, and the small starch granules. Satisfactory modification in malting should result in degradation of cell-wall material throughout the endosperm and release of starch and degraded protein during mashing. However, some areas of the endosperm (especially at the distal end) may contain undegraded endosperm cell walls in which starch extract can be trapped, and the trapped starch gives rise to glucan (gum) materials during mashing.

**Microbial Damage**

Cereal grains are important as food because of their excellent keeping qualities. Moisture content is the major factor in determining the storage behavior of grain, which is also influenced by temperature, oxygen supply, history and condition of the grain, length of storage, and biological factors (molds and insects). The respiratory rate of dry grain is low. As the moisture content is raised above 14%, the apparent respiration increases gradually until a certain critical moisture is reached above which respiration accelerates rapidly and the grain tends to heat. This sharp increase in respiration is due to the germination and growth of certain molds.

Fig. 8. SEM. a) Almost complete hydrolysis of cell wall and storage protein and extensive digestion of starch from region F of malt. b) Barley section from region F showing intact cell walls (CW), protein, and starch. c) High magnification of highly modified starch from region E of malt. d) Unhydrolyzed starch granules of barley from region E cut in half by razor blade during tissue preparation.
Fig. 9. TEM. a) Malt from region B showing most of cell wall (CW) digested, intact protein (P), partially digested small starch granules (S), and wedge-shaped digestion furrow in large starch granule (arrow). b) Intact cell wall (CW), starch (S), and protein (P) from barley in region B. c) Highly digested cell wall (CW) near scutellum (region E) of malt. d) Intact cell walls (CW) of barley from region E. e) Starch digestion (small starch granules (S); large starch granules (arrow) of malt from region B was minimal; as was protein (P) degradation. Cell walls (CW) were mostly removed.

Fig. 10. TEM. a) Intact protein (P) and starch (S) of barley from region B, representative of those components in various regions. b) Malt from region C showing peripheral digestion of starch (S) and some modification of protein (P). c) Digestion of starch from region E of malt (arrows). d) High magnification of digestion furrows (arrows) of starch granule (S) of malt from region E.
Y. Pomeranz

Fig. 11. Diagramatic summary of hydrolytic modification of cell walls, proteins, and starch in kilned barley malt.

(predominantly various species of *Aspergillus* and *Penicillium*) commonly found in soil and in previously used storage bins. Molds are invariably found on the grain and within the seed coats, even though the grain is harvested under ideal conditions.

Several investigations have indicated that the crease and palea are the primary site of infestation by microorganisms in oats (Pomeranz and Sachs, 1972). The area between the palea and the crease seems to be favorable for growth of microorganisms, where they are harbored in the mature and dried grain. Presence of a plaque with microbial growth (probably a slime-producing bacterial colony) beneath the palea of an oat kernel is indicated in Fig. 12a. Fungi under the hull of rice are shown in Fig. 12b.

### Instrumentation and Methodology

Much of the progress on relation between structure and end-use properties of foods has been made possible through advances in instrumentation and methodology (Pomeranz, 1976).

Scientists engaged in the study of materials have a large, increasing, and sometimes bewildering number of analytical techniques at their disposal (Williams and Goldstein, 1981). Significant among these are techniques that utilize electron beams as a source of information. Some of these techniques are compared in Table 4; many are highly complimentary. New methods eliminate (or at least reduce) artifacts from specimen desiccation and damage from excessive irradiation of biological systems. This has been accomplished along with increased sensitivity. Another biological application involves labeling with heavy atoms coupled to a particular chemical group of interest within a macromolecule or to a particular macromolecule in an assembly of macromolecules (Beer et al., 1981). Chemists who study solids recognize the complicated relationships between microstructure and properties of solids. High-resolution analytical electron microscopy is especially useful in studying those relationships.

Photoacoustic spectroscopy has found for quite a number of years important applications in characterization of solids, liquids, and gases from inorganic and biological sources. Recently, there has been interest in performing photoacoustics on a microscopic scale (Rosenwaig, 1979). Tsai et al. (1979) described the application of transmission scanning acoustic microscopy in nondestructive testing and evaluation. The instrument, used in the metal industry and in production of microelectronic computers, measures material parameters such as elastic modulus, mass density, and acoustic absorption rather than the index of refraction and optical absorption as observed in an optical microscope. The instrument does not require destructive sample preparation and unlike conventional optical microscopy, can illuminate and examine details deep below the surface of an opaque sample. Williams and Goldstein (1981) emphasized that while electron microscopy continues to develop and more advanced and higher resolution instruments become available, one of the regions of greatest promise is interfacing of computers to electron optical instruments. The exciting developments in this field will change the electron microscope, now predominantly an imaging instrument, into a data-generating instrument with concurrent imaging capabilities. For example, on-line microanalysis and particle size analysis can be performed on an SEM and AEM. Concurrent diffraction pattern analysis can also be performed with minimal operator intervention. Minicomputers can be used to control operating parameters, collect data, and assist in data interpretation (Stewart, 1981).

Fig. 12. a) Plaque with microbial growth on the inside of the palea of an oat kernel (20 μm marker); b) Fungi under the hull of rice (5 μm marker).
Table 4. Comparison of Techniques Utilizing Electron Beams (From Williams and Goldstein, 1981)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SEM</th>
<th>EPMA</th>
<th>TEM</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Specimen type</td>
<td>Bulk</td>
<td>Polished bulk</td>
<td>Thin section</td>
<td>Thin section</td>
</tr>
<tr>
<td>2. Image resolution</td>
<td>7-10 nm</td>
<td>7-10 nm</td>
<td>~0.2 nm (line)</td>
<td>~0.2 nm (line)</td>
</tr>
<tr>
<td>3. Chemical analysis</td>
<td>x-ray</td>
<td>x-ray</td>
<td>-</td>
<td>x-ray, energy loss</td>
</tr>
<tr>
<td>technique</td>
<td>EDS</td>
<td>EDS, WDS</td>
<td>-</td>
<td>EDS, CBD, EELS</td>
</tr>
<tr>
<td>a) measurement</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>equipment</td>
<td></td>
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</tr>
<tr>
<td>b) spatial resolution</td>
<td>~1 µm</td>
<td>~1 µm</td>
<td>~0.1%</td>
<td>~0.1%</td>
</tr>
<tr>
<td>c) minimum detectability</td>
<td>10^{-14}</td>
<td>10^{-15}</td>
<td>-</td>
<td>10^{-19}</td>
</tr>
<tr>
<td>d) minimum detectable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mass (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e) accuracy</td>
<td>-</td>
<td>±1% (rel.)</td>
<td>-</td>
<td>±5-10% (rel.)</td>
</tr>
<tr>
<td>4. Structural analysis</td>
<td>-</td>
<td></td>
<td>Elect.</td>
<td>Elect. diffraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>diffraction</td>
<td>diffraction</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>~1-5 µm</td>
<td>≤10-20 nm</td>
</tr>
<tr>
<td>5. Other techniques</td>
<td>Magnetic contrast</td>
<td>Electron beam induced conductivity</td>
<td>Channeling patterns (crystal orientation)</td>
<td>Voltage contrast</td>
</tr>
</tbody>
</table>

*AEM = analytical electron microscopy, CBD = convergent beam diffraction (microdiffraction), EDS = x-ray energy dispersive spectrometry, EELS = electron energy loss spectroscopy, EPMA = electron probe microanalyzer, SEM = scanning electron microscopy, STEM = scanning TEM, TEM = transmission electron microscopy, WDS = wavelength dispersive spectrometry (crystal spectrometer)*

What are the implications of those developments for the cereal chemist and technologist? I am confident that in the not too distant future we will witness adaptation of those instruments to biological systems, in general, and cereals, in particular. I am confident that we will be able to use the stage of the microscope to see, measure, record, and interpret:

- Structural features of the kernel as they relate to composition, hardness, resilience, conditioning, and milling;
- Changes that occur in a dough as it is mixed, fermented, proofed, heated, and baked;
- Changes in water migration, starch modification, and overall staling in a baked product as it is stored;
- Modifications in a germinating barley kernel as they relate to steeping, malting, mashing, and lautering;
- Extrusion of versatile products by energy-saving techniques;
- And many others.

I am confident that we will be in a position to put the micro hardness tester, the mixer, the oven, the extrusion equipment, the malting chamber or some of their adaptations on the stage of a microscope and follow continuously on a microscopic scale what is happening and learn to relate those microscopic changes to the real macro scale world.

As I have said on a previous occasion (Pomeranz, 1980), the four questions most commonly asked by workers in biological sciences are: what? how much? where? and what function? The question--what?--relates to the identity of components in the biological system under investigation. The answer to how much? provides information on the quantities of identified components in the system. The answer to the question where? is designed to localize the component(s) in the plant or animal tissue or in a processed food such as bread. The fourth question, has to do with function, either basic--physiological or applied--food processing. To what extent can answers to all four questions be generated by new instrumentation? I believe that recent developments show great promise in this respect.

Improving Nutritional Quality by Modifying Grain Morphology

The greatest promise, to my mind, lies not in applying instruments, but in restructuring the cereal grain. In recent years many studies have concerned the improvement of the nutritional value of cereal grains. Simple changes in grain morphology could be the basis of improvement. The embryo of cereal seeds is rich in protein (up to 38%), and the protein may contain about 7% lysine. Selection for larger embryos is particularly important if the whole seed (rather than starchy endosperm) is to be consumed. Variations in the
number of aleurone cells of the endosperm exist in corn, rice, and barley. The aleurone layer is rich in protein having a good amino acid balance. Selection for a high aleurone cell number could be useful, provided the high number is associated with improved nutritional value and the deleterious effects of high phytic acid in the aleurone layer are counteracted. Both in wheat and in rice, much of the protein is concentrated in the aleurone and outermost subaleurone. Those tissues are diverted to feed during milling and polishing of rice or during milling of highly refined wheat flour. "Restructuring" cereal grains for a more even distribution of protein throughout the whole endosperm would increase the protein content of milled products.

I am confident that by the time of the third millennium we will have as a result of a combination of classical plant breeding and modern genetic engineering a vastly improved grain. I am confident that modern genetic engineering, the precision tool of plant breeding, will be able to perform a microsurgery that will cut and paste together the genetic code of the improved cereal grain. That improved grain will be produced from high yielding cultivars, that are relatively insensitive to adverse conditions, make the best of sound cultural practices, and are uniquely structured to meet the specifications of millers, bakers, and makers. The spherical or barrel-shaped kernel with a minimum crease, will have an aleurone and germ positioned in the kernel in such a manner that it will make possible a flour extraction that approaches the starchy endosperm content. The milled product will, at least, equal in protein content the whole wheat and will be adaptable to production of a variety of cereal-based products. It will produce delectable, nutritious, and readily available foods that have excellent shelf life and meet with consumer acceptance.

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of year and station where grown on four Pacific Northwest wheat varieties. Am. Miller 65(7):34-36.


Wolf MJ, Seckinger HL, Rosewall EC, MacMasters MM, Rust CE. (1952). Studies on water-insoluble hemicellulloses of the endosperm cell walls in


Discussion with Reviewers

R. Moss: What is meant by endosperm cavity height? The distance within the endosperm, from the dorsal to the ventral side of the grain?
Author: I assume the author (Kosina, 1979) meant the distance within the endosperm, from the dorsal to the ventral side of the grain. The author, however, did not define the term endosperm cavity height in the paper.

E. Varriano-Marston: I do not see how protein body fusion can be significant in loaf volume potential since much of the literature suggests that it is the fibrillar proteins (gluten) that are important in breadmaking potential. Please explain; maybe I am reading you wrong.
Author: I did not say "identical with", I said "concomitant with" which is what our data show. "Limited aggregation" does not contradict involvement of "fibrillar" proteins; fibrillar proteins can aggregate.

Editor: How were the grain samples prepared for Scanning Electron Microscopy?
Author: Grain and malt samples were prepared for SEM by splitting with razor blades and mounting on specimen holders which were spread with a colloidal graphite adhesive. The specimens were then coated with a thin gold film in a sputter coater. Dough or bread samples are cut with a pair of sharp scissors with minimum distortion from smooth, freshly exposed surfaces. Small pieces are quick frozen and freeze dried and the freeze-dried pieces are broken - fractured to expose interior surfaces prior to coating.
SCANNING ELECTRON MICROSCOPY OF THE PERICARP AND TESTA OF SEVERAL SORGHUM VARIETIES

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Abstract

Pericarp thickness (determined by Z gene) varies greatly among sorghum varieties ranging from very thin (8 µm) to very thick (160 µm). Pericarp thickness also varies within an individual kernel. The areas below the style and near the hilum are the thickest with the sides of the kernel being thinnest. Scanning electron microscopy was used to document differences in pericarp thickness and to explain milling differences. Varieties with a thick pericarp had starch granules in the mesocarp cell layers. Sorghums with a thin pericarp did not have starch granules in the mesocarp except near the hilum and stylar area. U.S. sorghum varieties studied had a testa thickness of 16-40 µm (side of the kernel) but recently four Malian sorghums from a recent collection had very thin testae of 8-16 µm. The Sudanese sorghum Shawaya had a testa ranging in thickness from 28-40 µm.

Introduction

Sorghum (Sorghum bicolor (L.) Moench) is the major cereal grain consumed by many people in Africa and parts of Asia. Many sorghum food products are made from decorticated grain which has been ground into a flour. Pericarp thickness and presence of a pigmented testa affect the processing and nutritional properties of sorghum and sorghum products.

Kernel Structure

The sorghum kernel consists of three major areas: the pericarp, the germ, and the endosperm (Figure 1). The pericarp is derived from the ovary wall (Saunders, 1955). The pericarp layers include the epicarp, mesocarp, and endocarp (Figure 2). The epicarp, usually two or three layers thick, consists of long, rectangular cells which may contain pigments. The outer surface of the epicarp is usually covered with a waxy cuticle. Thickness of the mesocarp is determined by the presence or absence of starch granules in this layer. The endocarp layer includes the cross and tube cells whose major function is transport of moisture around the kernel. The endosperm consists of the aleurone layer, the peripheral endosperm, the corneous endosperm, and the floury endosperm. The endosperm texture in sorghum can be corneous, intermediate or floury depending on the ratio of corneous to floury endosperm.

Pericarp Thickness

Pericarp thickness is controlled by the 'Z' gene. A thin "pearly" pericarp results from the dominant Z gene (Z-) and a thick "chalky" pericarp from the homozygous recessive (zz) condition (Ayyangar et al, 1934). Pericarp thickness varies within individual kernels as well as among varieties. The mesocarp varies from a thin, almost nonexistent layer (thin pericarp), to several layers of starch-filled cells (thick pericarp). Variation in pericarp thickness among varieties occurs even when the homozygous recessive condition is present. Corneous sorghums with thick pericarps usually require decreased milling time and have slightly decreased yields of milled grain compared to corneous sorghums with thin pericarps (Da et al, 1982). Native women in Africa prefer thick, chalky sorghums for traditional milling with a wooden mortar and...
pestle. The thick pericarps are removed in larger pieces, thus requiring less deccorticating time. Since the pericarp breaks in the mesocarp layer (Freeman and Watson, 1969; Hubbard et al, 1950), the thicker pericarp (having thicker starchy mesocarps) break into flakes more easily. Using a specially modified Udy cyclone mill, G-766W, a sorghum with a thick pericarp, was shown to "peel" with large pericarp flakes produced, similar to what is seen with the traditional mortar and pestle milling system (Shepherd, 1979; Shepherd, 1981).

Pericarp thickness affects grain weathering (pre-harvest deterioration). Sorghums with thick pericarps generally appear to deteriorate more rapidly than sorghums with thin pearly pericarps. Perhaps the starch granules in the mesocarp provide a readily available substrate for the fungi (Leukel and Martin; 1943; Ellis, 1972; Glueck, 1979).

Presence of Testa

Two genes, B1 and B2, control the presence or absence of a pigmented testa in sorghum. If either the B1 or B2 gene is homozygous recessive, the pigmented testa does not form and is thought to be absorbed (Swanson, 1928; Quinby and Martin, 1954). During kernel development, the cells of the inner integument are crushed to form a single, often continuous, layer called the testa. Morrall and coworkers (1981) studied the development of the testa and reported that membrane-bound vesicles in the inner integument cells became filled with what was presumably tannin. During the milk dough stage the central vacuoles of the inner integument cells expanded to fill almost all of the cells and during the soft dough stage were filled with tannin. The testa is between the tube cell layer (innermost part of the pericarp) and the aleurone layer (outermost part of the endosperm). Remnant cells from the inner integument are sometimes observed in those kernels in which the pigmented testa is absent. Ayyangar and Krishnaswami (1941) reported the presence of a colorless layer between the pericarp and aleurone layer which they called an unpigmented testa.

Testa Thickness

Differences in thickness of the testa within individual kernels occur. The thickest area is below the style (100-140μm) and the thinnest on the sides of the kernel (means of 25 and 18μm on hilar side and opposite side) (Blakely et al, 1979). A partial testa has also been observed with the pigmented testa present only in some areas of the kernel (Blakely et al, 1979). Several varieties of local sorghums from Mali with very thin testae have been described by Dr. John Scheuring (private communication). Blakely et al (1979) also reported that testae often appeared as two overlapping layers, often of different colors. This layering of the testa can be seen in Figure 3-D.

The presence of a pigmented testa in sorghum adversely affects the nutritional value of sorghum food and feed products. High levels of tannins are found in sorghums with a testa which depress feed intake and feed efficiency (Featherston and Rogler, 1975; Maxson et al, 1973; Cousins et al, 1981). The levels of tannins and other polyphenols in the testa and pericarp are affected by the B1 and B2 genes. When these two genes are dominant and a spreader gene (S) is dominant, the pericarp has high levels of polyphenols and appears brown in color. The intensity of the brown pigmentation is affected by the genetic pericarp color, whether red or white. An additional set of genes (Tp) controls the pigmentation of the testa. The
Fig. 3. Scanning photomicrographs depicting pericarp and testa thickness of several sorghum kernels.

A) Thin pericarp without testa (IS9986)
B) Thick pericarp without testa (Kende - Mali)
C) Thin pericarp with thin testa (Experimental line, College Station, Texas)
D) Thick pericarp with thin testa (Kende - Mali)
E) Thin pericarp with thick testa (GA615)
F) Thick pericarp with thick testa (ATx623 X SC0103)

P - pericarp, Al - aleurone layer, T - testa, SG - starch granule, M - mesocarp

The sorghum kernels were longitudinally cut and photomicrographs taken on the sides of the kernels in similar areas on each kernel so that differences in pericarp and testa thickness could be minimized.
The testa is usually brown (Tp) but it can be purple (tptp) (Casady, 1975). The phenolic compounds in the testa impart some resistance to field fungi thus decreasing susceptibility to pre-harvest deterioration (Glueck, 1979).

When sorghum with a testa is milled for use in food products a dark or speckled flour is produced, causing undesirable color in ogi and tortillas, two African food products (Akingbala et al, 1981; Da et al, 1982). When alkali is used to process tortillas and tortillas, color changes become even more pronounced (Khan et al, 1980; Johnson, 1981). These color changes are caused by the polyphenols in the pericarp and testa. Presumably, sorghums with thin testae would have lower concentrations of phenolic compounds, thus decreasing the color problem in food products, but might retain some resistance to grain deterioration. Also a thin testa may be easier to remove during milling.

Since pericarp thickness and presence of a pigmented testa affect the processing of sorghum products, the objective of this work was to document genetic related differences in sorghum pericarp thickness and how those differences affect milling characteristics.

Materials and Methods

Samples

Guineense sorghums with thick and thin pericarps, Nio-Fionto, and four thin testa varieties were grown in Mali in 1981. Kernels selected from the F2 population of BTx623 X CS3541 were harvested in 1980 from College Station, Texas. The following six sorghums were selected to demonstrate differences in thin and thick pericarps and testae: a white sorghum with a thin pericarp without a pigmented testa (IS9985), a white Malian Kende sorghum with a thick pericarp and no pigmented testa, a red sorghum with a thin pericarp and thin testa (experimental line- College Station, Texas), a white Malian Kende sorghum with a thick pericarp and thin testa (CSM-184), a brown sorghum with a thin pericarp and thick testa (GAG615), and a brown sorghum with a thick pericarp and thick testa (ATx623 X SC0103- Sel). The Shawaya sorghum with an extremely thick pericarp was received from Sudan in 1981.

Scanning Electron Microscopy

Kernels were cut in half with a razor blade and oven-dried at 45°C overnight. Samples were mounted on aluminum stubs with silver conductive paint and coated with a 200 A layer of gold-palladium prior to viewing. Samples were examined with a JEOL JSM35 Scanning Electron Microscope with an accelerating voltage of 20kV. Care was taken to view and photograph all specimens from the same angle so as to minimize artificial distortion of pericarp thickness.

Milling

Traditional mortar and pestle milling by a Malian woman was used to mill 3 types of Malian sorghums as described in the Results and Discussion section.

Results and Discussion

Screening of sorghum samples indicated a large variation in pericarp thickness (Table 1). York (1977) proposed that the genes denoting pericarp thickness be reclassified to reflect his belief that genes other than the Z locus influence the thickness of the starchy mesocarp.

The thin pericarps contained few, if any, starch granules (Figures 3-A,C,E), whereas thick pericarps contained abundant starch granules in the mesocarp area (Figures 3-B,D,F). When present in the thin pericarp, starch granules were in the mesocarp below the style and around the hilum area which are the two thickest parts of the pericarp. The ranges in pericarp thickness observed in these and other samples are reported in Table 1. The upper value of the thin and intermediate pericarps was that measured below the style and next to the hilum.

Table 1. Pericarp thickness of sorghum varieties

<table>
<thead>
<tr>
<th></th>
<th>Thin</th>
<th>Intermediate</th>
<th>Thick</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-32um</td>
<td>28-48um</td>
<td>40-160um</td>
<td></td>
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</table>

The testa in Figure 3-C was thinner (16um) than in the varieties in Figures 3-E and F (32um), but it still was not as thin as the Malian sorghum in Figure 3-D (8um). The Malian sorghums had much thinner testae than those of the U.S. sorghums studied.

Pericarp Thickness Among Varieties

Photomicrographs were taken of sorghum kernels from the F2 population of the cross BTx623 X-CS3541 which were segregating for pericarp thickness. All the F2's had white pericarps without pigmented testae. Some of the progeny had a thin pericarp without starch granules in the mesocarp resembling the parent CS3541 (Figure 4-A); another had a thick pericarp with considerable starchy mesocarp resembling the parent BTx623 (Figure 4-C); the last F2 had an intermediate pericarp thickness (Figure 4-B) with relatively few starch granules in the mesocarp. All photomicrographs were taken at the sides of the kernel in the thinnest areas of the pericarp in approximately the same place on each kernel.

Pericarp Thickness Within Kernel

Photographs of Nio-Fionto grain were taken at different points around the periphery of the kernel to observe variations in pericarp thickness within individual kernels. The pericarp next to the hilum (80 um) (Figures 5 (point D) and 6-D) was the thickest, with that near the top of the kernel (64 um) (Figures 5 (point B) and 6-B) and near the style (56 um) (Figures 5 (point A) and 6-A) intermediate, and that at the side of the kernel the thinnest (48 um) (Figures 5 (point C) and 6-C). The waxy cutin layer on the kernel next to the style is much thicker than in other areas of the pericarp. Pericarp thickness measurements did not include the cuticle. These data suggest that it is possible to compare pericarp thickness among sorghum varieties only if the comparisons are based on the examination of similar locations on the kernel periphery.

Pericarp Thickness and Milling

When sorghum is milled, the endosperm type...
SEM of Sorghum Pericarp and Testa

Fig. 4. Scanning electron photomicrographs of sorghums with varying pericarp thickness. Samples were the F2 population from the cross BTx623 (thick pericarp) X CS3541 (thin pericarp). A) Thin pericarp B) Intermediate pericarp C) Thick pericarp

Fig. 5. Approximate location on kernel where Figure 6 photomicrographs were taken.

(percentage of corneous or floury endosperm) influences how well the kernel can be decorticated. More kernel breakage and incomplete milling occur in varieties with more floury endosperm (Maxson et al., 1971). The following milling trials were made with corneous to intermediate texture sorghums to permit comparisons based on pericarp thickness.

Milling trials performed on several sorghum samples from Mali and Upper Volta showed large differences in milling times between the thin and thick Guineense and the very thick Nio-Fionto grains (Table 2). Using a wooden mortar and pestle, average milling times for 2 kg lots of grain were 27.7, 19.8, and 11.0 minutes for the thin Guineense, the thick Guineense, and the Nio-Fionto, respectively. The milling was performed by native Malian women with three replications for each of four Guineense varieties and eight replications for the very thick Nio-Fionto sorghum. In Figure 7, differences in mesocarp thickness of these varieties is evident. Photographs B, D, and F were taken at approximately the same place on the kernels as indicated by the arrows in A, C, and E. The thin Guineense has virtually no mesocarp. The thick Guineense has a starchy mesocarp. The Nio-Fionto grain also has a thick, starchy mesocarp; the mesocarp is a layered structure with abundant starch granules. The photographs illustrate that the very thick, layered pericarp of the Nio-Fionto sorghum would easily be torn or peeled from the endosperm, thus decreasing milling time with the traditional pounding method of milling.

Testa Thickness Among Varieties

Variations in testa thickness were studied using four Malian sorghums and one (Shawaya) from Sudan. There was considerable variation in testa thickness among the Malian varieties (8-16μm) (Figures 3-D, 8-A,B,C). However, the testae of these varieties were thinner than those of the other varieties studied (e.g. Figures 3-E and 3-F). The opposite extreme was seen with the Sudanese Shawaya (testa thickness 24-40μm) (Figure 9). This grain has a thick testa, the thickest pericarp encountered in our studies (160μm). It was approximately 3x the thickness...
Table 2. Average milling time and endosperm recovery of sorghums with different pericarp thickness

<table>
<thead>
<tr>
<th>Grain Type</th>
<th>Pericarp Thickness</th>
<th>Average Dehulling Time (minutes)</th>
<th>Endosperm Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nio-Fionto</td>
<td>Very thick</td>
<td>11.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malian Guineense (thin pericarp)</td>
<td>Thin</td>
<td>26.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>68.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malian Guineense (thick pericarp)</td>
<td>Thick</td>
<td>19.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Voltaic Guineense (thin pericarp)</td>
<td>Thin</td>
<td>29.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Voltaic Guineense (thick pericarp)</td>
<td>Thick</td>
<td>20.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup>NA - data not available.

Numbers in same column with different superscripts are significantly different (P > .05)

Fig. 6. Scanning electron photomicrographs of Nio-Fionto sorghum at several locations around kernel periphery depicting variation in pericarp thickness. Thickness of the pericarp at the following locations was:

A) Below style - 56µm
B) Near top of kernel - 64µm
C) Side of kernel - 48µm
D) Near hilum - 80µm

Al - aleurone layer, S - style, C - cuticle, Ep - epicarp, M - mesocarp
Fig. 7. Scanning photomicrographs of three sorghums from Mali.
A&B) Thin pericarp (Guineense)
C&D) Thick pericarp (Guineense)
E&F) Nio-Fionto grain with a much thicker pericarp and considerable starch in the mesocarp
P - pericarp, Al - aleurone layer. The arrows on A, C, E indicate where B, D, F sections were photographed.
Fig. 8. Three Malian sorghums with thin testae. P - pericarp, T - testa, Al - aleurone layer

Fig. 9. Shawaya sorghum from Sudan with an extremely thick pericarp and a thick testa. P - pericarp, T - testa, Al - aleurone layer
of other thick pericarp sorghums studied.

The thin testa Malian varieties would be desirable in food products utilizing the entire ground kernel. Milling studies need to be performed before any conclusions can be drawn concerning the ease of removal of the testa from these varieties.

Based on the thickness of its pericarp, Shawaya would appear to be ideally suited to traditional milling methods. However, the grain is black in color. Shawaya may be a genetically red sorghum with a photosensitive pigment in the epicarp. Unless the pericarp and thick testa were removed prior to processing, the benefits of ease in milling would be outweighed by the development of undesirable colors. The very thick pericarp would also result in lower yield of milled grain.

Conclusions

Pericarp and testa thickness varies among sorghum varieties ranging from very thin to very thick. These differences affect the milling properties of the grain. Pericarp thickness affects mechanical and traditional hand milling systems differently. Our microscopic techniques can be used by plant breeders to select for pericarp and testa thickness.

Acknowledgements

The authors wish to thank Dr. John F. Scheuring, with ICRISAT in Mali, for use of the milling data and for the sorghum varieties used in the study of pericarp thickness and thin testa varieties. This research was supported in part by Grant AID/DSAN/XII/G-0149 from the Agency for International Development, Washington, DC 20523.

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All Texas A&M University theses and dissertations are available on loan from TAMU Library Interlibrary Loan, College Station, Texas.

Discussion with Reviewers

C.W. Glennie: Did you observe any difference in the size of the starch granules in the pericarp (mesocarp) as compared to those in the endosperm?

Authors: There appears to be a large difference in the sizes of the starch granules in the two areas. The starch granules in the pericarp are approximately 2-8 μm while the starch granules in the endosperm vary depending on whether in the peripheral endosperm (6-10 μm), the corneous endosperm (7-13 μm), or the floury endosperm (13-30 μm). There is a large variation in size of sorghum starch granules. These are some general values I have selected and by no means are they absolute values.

C.W. Glennie: Would the authors care to comment on their suggested photo-sensitive pigment mentioned in the last paragraph of Results and Discussion?

Authors: At this point we believe that the pigment is an anthocyanin. The young seed is red and gradually becomes darker, in some cases black. The same purplish-black color has been attributed to anthocyanins in the leaf-sheath and glume of some sorghum varieties. We presently have some crosses in the green house with these black seeded varieties and hope to get enough seed to do some extraction and identification of the flavonoid compounds soon.

C.W. Glennie: Were all testae observed as two cells thick?

Authors: No, they were not. The sorghums with testae seem to fall into two categories: 1) those sorghums with a red, white, or yellow pericarp with pigmented testae and 2) those sorghums with pigmented testae and a dominant spreader gene causing the phenotypic pericarp color to be brown, regardless of the genetic color of the pericarp (white, yellow, or red). The sorghums in the first group appear to have the two layerd testae usually two different colors. The sorghums with the pigmented testae and spreader appear to have a more continuous testa layer. We are presently doing some additional microscopy work looking at sorghums with testae to see if the two-layer testa phenomenon holds true only for the one type of sorghum.

A. Shepherd: Is testa thickness chosen because of its ease of measurement? One might think that tannin content or some other measure of pigmentation would be more relevant. Please explain the choice. Do you have any hope of finding a single gene responsible for testa thickness?

Authors: It is quite possible that there is a single gene responsible for testa thickness. Until we received the thin testa Malian sorghum varieties and the thick testa Sudanese Shawaya we had seen very little difference in testa thickness among varieties so no work has been done to identify a gene for pericarp thickness.

We undertook this work to verify that the Malian sorghums did indeed have thinner testae than the U.S. sorghums we have studied. The initial quantity of samples available was very small, thus we have not yet been able to do any tannin analyses.

E. Varriano Marston: Why did you oven-dry the kernels before viewing in the SEM? The samples should be sufficiently dry without drying any further. At what temperature were they dried?

Authors: The humidity here is so high that we cannot coat the kernels until the samples are dried to reduce the excess moisture. They were dried at 45°C.

Editor: What do a, b, c, d in Table 2 signify?

Authors: These are referenced by the superscript 1. This is the usual way of referencing means that are significantly different as determined by Duncan’s Multiple Range Test.
THE MICROSCOPIC STRUCTURE AND CHEMISTRY OF RAPESEED AND ITS PRODUCTS

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Abstract

The location and distribution of some of the storage constituents in the structures of rapeseed and its products were investigated. Hand-cut or glycol methacrylate-embedded sections were stained with dyes or fluorochromes of known specificities and examined using fluorescence, bright-field and/or polarizing microscopy. Results obtained from the study were based upon observations of characteristics of the various stainings, birefringence, induced fluorescence and autofluorescence. The effects of enzymatic hydrolysis, solvents and processing on the cellular structures and their affinity for certain dyes/fluorochromes were also investigated. Major and minor storage constituents were tentatively located in the structure of rapeseed. Lipids and proteins were stored within separate cellular organelles which were distributed throughout the aleurone layer of the endosperm and cells of the embryo. These two accounted for the major portions of rapeseed storage reserves. Phytin crystals were detected inside the protein bodies of the embryonic cells. Most of the rapeseed polysaccharides were present as structural (cell-wall) carbohydrates of which amyloid was one of the major components. The testa of the yellow seed-coated cultivar, Candle, was structurally and chemically different from that of other rapeseed varieties.

Introduction

Economically, rapeseed is the most important oil seed crop in Canada. Canadian rapeseed (commonly known as Canola) is low in erucic acid and glucosinolate, and supplies 50% of the total domestic edible oil market. A certain portion of its byproduct, rapeseed meal, has a high nutritional value and is used as livestock and poultry feed. In addition to 37-45% oil, rapeseed contains 20-25% protein (rich in lysine, methionine and cystine), minerals such as calcium, iron, magnesium and phosphorus, and vitamins, particularly niacin (Clandinin 1981). All of these components are stored in the seed in highly compartmented, morphologically discrete forms. A thorough understanding of the location and distribution of these storage reserves undoubtedly would be of benefit in improving processing techniques which in turn lead to increased quality and nutritional value of rapeseed products.

Recent studies on rapeseed ultrastructure using electron microscopy (EM) and x-ray microanalysis have provided useful information regarding the cellular location of storage and mineral reserves (Hofsten 1974a and b, Mills and Chong 1977, Stanley et al 1976). While both transmission EM and scanning EM have revealed high resolution details of the fine structure of rapeseed, they provide only minimal chemical information. Specific EM markers for the numerous storage reserves in seed tissues are rare. In contrast, a wide range of highly sensitive staining techniques is available for detecting major and minor storage reserves in seeds (Fulcher and Wong 1980). Most of these methods employ very sensitive and specific fluorescent markers with the potential to detect substances in concentrations as low as 10-18 moles (Von Sengbusch and Thaer 1973). The sensitivity and specificity of fluorescence microscopy makes it an indispensable tool for studying the location and distribution of various storage reserves in rapeseed.

Materials and Methods

Rapeseed Cultivars

Mature seeds of Brassica campestris L. (cultivars: Echo, Candle and Sarson R500) and
Brassica napus L. (cultivars: Altex and Tower) were obtained from Dr. R. Loiselle of Resources of Canada, Ottawa Research Station, Agriculture Canada.

Hand-cut Sections

For rapid analysis of seed tissues, and in order to avoid extraction or modification of certain seed compounds (e.g., phenolics, lipids) during normal fixation and embedding procedures, hand-cut sections were examined routinely in parallel with fixed and embedded tissues. Sections 10-20 μm thick were cut using a clean, toluene-washed razor blade and placed on glass slides for immediate examination. Although hand-cut sections do not permit the high resolution which is characteristic of thin plastic sections, recent improvements in microscope design (notably fluorescence epi-illumination) allow dramatic improvements in resolution of components in thick sections. Hand-cut sections can be obtained from most seed tissues with a minimum of practice. Glycol Methacrylate (GMA)-embedded sections. Hand-cut sections were examined routinely in parallel with fixed and embedded tissues.

Glycol Methacrylate (GMA)-embedded Sections

For routine high resolution work, seed tissues were fixed and plastic-embedded using procedures described by Fulcher and Wong (1980). Briefly, tissues were fixed in 3% glutaraldehyde in 0.025M phosphate buffer, pH 7.2 at 4°C for 48 hr, dehydrated through methyl cellosolve, ethanol, m-propanol and n-butanol and infiltrated with GMA monomer (Feder and O’Brien 1968) for 3-5 days prior to polymerization at 60°C in gelatine capsules. Alternatively, to minimize loss of solvent- and plastic-soluble components (especially storage lipids), fixed tissues were embedded in a glutaraldehyde-urea-glycol methacrylate mixture (Pease 1973). This modified GMA procedure has been described by Hargin et al. (1980).

Sections were cut 1-5 μm thick using glass knives and affixed to glass slides for examination.

Microscopic Examination

Sections were examined with a Zeiss Universal Research Microscope (Carl Zeiss Ltd., Montreal) equipped with both a conventional bright-field illuminating system and a III RS epi-illuminating condenser combined with a HBO 200 W mercury-arc illuminator for fluorescence analysis. The III RS condenser contained three fluorescence filter combinations (FC I, II and III) each with a dichromatic beam splitter and an exciter/barrier filter set with maximum transmittance at 365 nm/>418 nm (FC I), 450-490 nm/>520 nm (FC II) and 546 nm/>590 nm (FC III). Photomicrographs were obtained using 35 mm Kodak Ektachrome 400 Daylight film. Specimens were photographed unstained (to demonstrate autofluorescent substances or after one or more of the following staining procedures.

(a) Phenolics. Unstained sections were mounted in oil and examined microscopically using filter system FC I. Many phenolic compounds emit blue autofluorescence under short wavelength excitation. To enhance autofluorescence, sections were placed inside a chamber saturated with ammonia vapor. They were then mounted in oil and examined immediately. Alternatively, sections were stained with 0.05% Toluidine Blue O (Sigma Chemical Co., St. Louis, Mo., C.I. 52040) in benzoylate buffer, pH 4.4, according to the method of Feder and O’Brien (1968). Stained sections were rinsed in water and water-mounted, or air-dried and oil-mounted, prior to examination in bright-field optics.

(b) Carbohydrates. Sections were stained with one of the following agents. Control sections were viewed without staining. Periodate-Schiff’s (PAS) reaction to detect periodate-sensitive carbohydrates: Sections were first treated with saturated 2, 4-dinitrophenyldrazine (Calbiochem, San Diego, Calif.) in 15% acetic acid for 10 minutes to block native or fixative-induced Schiff’s-positive aldehydes and were then oxidized with 1% periodic acid (w/v in water) for 10 minutes. After thorough washing with water (5-10 min.), oxidized sections were treated with Schiff’s reagent (Fisher Scientific Co., Fair Lawn, N.J.) for 1-2 minutes. Sections were rinsed with water before microscopic examination using the FC III filter system.

Hydrogen sulfite test. Sections were stained 1-2 minutes in 0.001% (w/v) aqueous Calcofluor White M2R New (American Cyanimid Co., Bound Brook, N.J.). After rinsing in water they were air-dried, mounted in oil, and examined for fluorescence using FC I.

Congo Red. Sections were stained 10-20 minutes in 0.001% (w/v) aqueous Congo Red (Fisher Scientific Co., Fair Lawn, N.J.; C.I. 22120). They were then rinsed in water, air-dried, and mounted in oil for fluorescence examination using FC III.

Siddiqui and Wood (1971) have demonstrated a water-soluble amyloid in rapeseed meal. In order to identify the tissue distribution of rapeseed amyloid, several hand-cut seed sections were extracted with hot (95°C) distilled water for up to 3 hr. Extracted and unextracted (control) sections were then stained by the methods outlined above.

(c) Storage Proteins. Rapeseed proteins were detected by staining sections with ANS (1-anilino-8-naphthalene sulfonic acid) or Acid Fuchsin as described by Fulcher and Wong (1980). ANS imparts intense blue fluorescence to storage protein bodies when viewed with FC I, Acid Fuchsin imparts red fluorescence when used with FC III.

(d) Phytin. Rapeseed phytin inclusions were demonstrated by flooding tissue sections for 1-2 minutes with freshly-prepared 1% (w/v) aqueous Alizarin Red S (Aldrich Chem. Co. Inc., Milwaukee, Wis., C.I. 58005) containing 1% (w/v) ammonium hydroxide at pH 6.2-6.5 (Pearse, 1972). Stained sections were rinsed briefly and mounted in water and examined using bright field optics. For improved sensitivity and resolution of phytin reserves, sections were then mounted in oil and examined for 15 minutes in 0.01% (w/v) aqueous Acriflavine-HC1

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Microscopic Structure and Chemistry of Rapeseed

(Matheson, Coleman and Bell Manufacturing
Chemists, Norwood, Ohio) at pH 3.1 using the
method of Tanke and Van Ingen (1980) but without
prior acid hydrolysis. Treated sections were
washed with ethanol, air-dried and mounted in
oil for fluorescence microscopy using FC III.

The crystalline nature of the phytin
reserves was confirmed by examining sections
with polarizing optics. Some sections were also
treated with a crude phytase preparation by
incubating the sections in 0.2% (w/v) wheat
phytase (Sigma Chemical Co., St. Louis, Mo.) in
0.1 M sodium acetate buffer (pH 6.8) at 55°C for
24 hr. Digested sections were rinsed extensively
in water and then examined with polarizing optics
or in bright field after staining with Toluidine
Blue O (as mentioned before) or Alizarin Red S.
Control sections were incubated in acetate buffer
only prior to staining.

(e) Storage Lipids. Major storage lipid reserves
were detected using methods described by Fulcher
and Wong (1980). Hand-cut or modified GMA-
sections were stained with 0.01% (w/v) aqueous
Nile Blue A (Eastman Kodak Co., Rochester, N.Y.
C.I. 51180) for 60 seconds. Stained sections
were washed and mounted in water under a cover
slip and examined microscopically using filter
system FC II. Control sections were viewed
without staining or were extracted with
several changes of hexane for 10-20 minutes
before staining.

Results and Discussion

General Morphology of Rapeseed

Rapeseeds are globose in shape and range in
size from 1.3 to 2.5 mm in diameter. The seed
coat may be black, brown, or yellow, depending
on the variety, and the surface of the seed is
fairly reticulated. Like many dicotyledonous
seeds, the embryo comprises a large proportion
(as much as 80%) of the mature seed, and
consists of a radicle and two conduplicate
cotyledons. The majority of the cells in the
embryo are storage parenchyma cells containing
the major reserves of protein, lipid, carbohydrate and phytin. Complete descriptions of the morphology of the mature rapeseed have been presented by Winton and Winton (1932) and Vaughan et al (1976).

The Seed Coat

The embryo is enclosed by a complex seed
ccoat containing an inner, single-cell layered
endosperm (or aleurone layer) and an outer,
multilayered testa. The latter contains an
epidermal layer, subepidermal layer, a
distinctive palisade layer, and a pigment layer
(see Fig. 1, and Winton and Winton 1932).

Microchemical methods are particularly
useful in demonstrating major differences in
seed coat composition and structure between
rapeseed varieties. For example, the palisade
cells of dark-coated varieties such as Tower
stain a deep green color with Toluidine Blue
(not shown) while yellow varieties such as
Candle show very little if any green coloration
after Toluidine Blue. The characteristic green
color is a well-known empirical indicator of
lignins and other phenolics (Feder and O'Brien
1968; Fulcher et al 1972), and the observation
that dark-colored cultivars are typically high
in lignin while yellow cultivars are low
(Theander et al 1977) corresponds to observed
differences in Toluidine Blue staining. There
may also be differences in seed-coat auto-
fluorescence characteristics among varieties.
In most varieties, both the pigment strand
(between the palisade and aleurone cells), and
the outer epidermal and subepidermal layers, are
intensely autofluorescent using FC I (Fig. 1).
However, in some yellow-coated varieties (e.g.
Candle), pigment layer autofluorescence is not
detectable. Although the identity of the
autofluorescent material is not known, blue
autofluorescence is also a characteristic of
many low molecular weight plant phenolics
(Fulcher et al 1972; Harris and Hartley 1976)
and may be useful in locating certain classes of
phenolic residues in seed coat tissues.

Of particular concern to rapeseed
processors is the presence in some cultivars of
the so-called seed-coat mucilage, a very
hydrophilic polysaccharide which interferes with
some processing systems because of its
characteristics. For example, upon hydration of
the seed coat of Candle seeds, a pronounced,
swollen mucilaginous layer forms on the outer
surface of the coat as shown by Van Caesselle
et al (1981) using bright field staining
methods. Fluorescence methods employing Congo
Red also provide especially sensitive means for
high resolution detection of seed coat mucilage
in situ, as shown in Fig. 2, which illustrates
the thick mucilaginous layer on the outer
surface of Candle seed. The thick layer of
mucilage is completely absent from most other
varieties, such as Echo (Fig. 1).

Figures 1 and 2 illustrate three features
of fluorescence microscopy which offer
advantages over bright-field approaches to seed
analysis. First, the instrument frequently
demonstrates compounds or structures which are
autofluorescent but which may not be stainable
or visible using other conventional means (as
in the pigment layer shown in Fig. 1). Second,
it is frequently possible to combine two or
more fluorescent techniques to allow
simultaneous detection of several compounds (as
in Fig. 1, which shows autofluorescence patterns
as well as the distribution of Congo Red-stained
materials). Third, and perhaps most important,
the fluorescence approach is particularly
useful for high resolution evaluation of
materials which would otherwise be difficult to
detect by bright field staining methods. Figure
2, for example, shows very high contrast of
Congo Red stained material in the seed coat - in
an equivalent, thin (1-3 um) plastic section
stained with Toluidine Blue, the mucilage
deposits were virtually impossible to detect
due to low contrast. Calcofluor White M2R
New reacts in a manner similar to Congo Red,
in also having an affinity for mucilage layers.
However, its fluorescence characteristics
(bright blue) are very similar to those of
autofluorescent structures in the seed coat.
(e.g. the pigment layer) and it becomes difficult to differentiate autofluorescence from Calcofluor fluorescence. Thus, Congo Red is the preferred fluorochrome for seed coat studies.

Cell Wall Carbohydrates

Unlike cereal grains, rapeseed contains only small amounts of starch; most of the rapeseed carbohydrates occur as structural (cell wall) polysaccharides in the embryo. A small number of starch grains can be demonstrated in the cotyledons using the PAS reaction or by staining with IKI.

Application of the optical brightener Calcofluor White M2R New is a particularly useful procedure for fluorescent staining of embryo cell walls. The reaction is rapid, provides high contrast, and imparts intense fluorescence to all cotyledonal cell walls (Fig. 3). Although the identities of the cell wall polysaccharides which are responsible for Calcofluor binding have not been determined, the dye has demonstrated an affinity for glucose-rich polymers (particularly β-glucans) such as oat β-glucan, lichenan, hydroxethyl cellulose, tamarind amyloid (Wood and Fulcher 1970) and cellulose fibers (Hughes and McCully 1975). Recent in vitro analyses have also demonstrated a marked interaction between Calcofluor and a water-soluble amyloid extracted from de-hulled rapeseed (P.J. Wood, personal communication). A comparison of hot water-extracted and unextracted hand-cut sections followed by Calcofluor staining demonstrated a marked reduction in cell wall staining in the extracted sections. These observations suggest that rapeseed amyloid occurs primarily in the embryo cell walls, but it must also be noted that other cell wall components, such as cellulose, may also be responsible, in part, for the notable affinity of Calcofluor for the cell walls. Congo Red can be used interchangeably with Calcofluor for routine demonstration of rapeseed embryo cell walls.

Storage Protein Bodies

The most abundant structures in rapeseed cells are storage protein bodies. They occur in both the aleurone layer and most of the cells of the cotyledons and radicle. Typically, they are 1-10 μm in diameter and may be roughly spherical or angular in shape, as shown in Fig. 4. Both ANS (Fig. 4) and Acid Fuchsin provide intense fluorescent staining of storage protein bodies and may be used to advantage in high resolution analyses. ANS provides blue fluorescence under excitation using FC I. Further discussion regarding the application of these fluorochromes in seed analysis is available elsewhere (Fulcher and Wong 1980). Because both fluorochromes are reactive only with protein bodies, they are particularly useful in unequivocal identification of storage proteins in most rapeseed products, including meal and protein isolates.

Phytin

With the exception of the aleurone layer, most, if not all of the protein bodies in other tissues contain several small (0.5-2.8 μm diameter) spherical inclusions which are unstained by Acid Fuchsin or ANS. These are the phytin globoids which may account for up to 10% of the dry weight of cruciferous seeds (Hofsten 1973). Phytin, or myo-inositol hexaphosphate, is typically present in rapeseed cells in the form of crystalline salts containing calcium, magnesium, and potassium, as shown by x-ray microanalysis (Hofsten 1974a; Mills and Chong 1977). Hence, crystalline globoids are readily detected as anisotropic structures using polarizing optics. They are also similar to cereal phytin globoids in acquiring a distinctive pink coloration after staining with Toluidine Blue (Fulcher et al 1980). Like barley globoids (Jacobsen et al 1971), rapeseed phytin crystals are completely removed from sections using crude phytase preparations.

Unfortunately, these methods do not provide entirely reliable markers for phytin globoids in situ. For example, other seed deposits, such as occasional small starch grains, are also birefringent, and the identification of phytin crystals is even more uncertain using polarizing optics to examine highly disrupted material such as rapeseed meal and flours in which minute cell wall particles may also be birefringent. Toluidine Blue imparts only low contrast in thin sections used for high resolution microscopy, and the metachromasia which is characteristic of Toluidine Blue is dependent upon an ill-defined degree of hydration of sections, which makes consistency rather difficult. However, these methods demonstrate the histochemical

Figure 1. Congo Red-stained GMA section of Echo rapeseed showing blue autofluorescence of the sub-epidermis (SE) and pigment layer (PL) as well as minor Congo Red staining in the cell walls (arrows) of the aleurone layer (A). The palisade cells (PC) are neither autofluorescent nor notably stained with Congo Red. Photographed using FC I. Figure 2. Congo Red-stained section of Candle rapeseed showing extensive mucilage layer (M) on the seed surface (A = aleurone layer; PC = palisade layer). Photographed using FC III. Figure 3. Calcofluor White-stained hand-cut section of Candle showing pronounced fluorescent staining of cotyledon cell walls. Photographed using FC I. Figure 4. ANS-stained GMA Section of Echo showing pronounced fluorescent staining of protein bodies (arrows) in both the aleurone layer (A) and cotyledon parenchyma (C). Photographed using FC I. Figure 5. Acriflavine HCl-stained GMA section of Altex showing bright red phytin globoids (arrows) as they occur within protein bodies of cotyledon sections. Photographed using FC III. Figure 6. Nile Blue A-stained hand-cut section of Echo showing yellow fluorescent lipid reserves in the aleurone layer (A) and cotyledon (C). Photographed using FC II. Figure 7. Nile Blue A-stained, modified GMA section of Altex showing lipid reserves (yellow fluorescence) and protein bodies (*) in the cotyledon. Photographed using FC II. Figure 8. Nile Blue A-stained, hand-cut section of commercial rapeseed meal showing residual lipid reserves (yellow fluorescence). Photographed using FC II. (Scale bars on each micrograph represent μm.)
Microscopic Structure and Chemistry of Rapeseed

similarity between rapeseed and cereal globoids, and they are certainly very useful for examining relatively thick (5-20 μm) embedded or hand-cut sections. In order to both the sensitivity and speed of phytin detection, we have adopted the fluorescent marker, Acriflavine-HCl for routine high resolution use. The marker is extremely sensitive, and although the mechanism of its interaction with phytin is unknown, it imparts intense red fluorescence only to phytin globoids with any of the three fluorescence filter combinations (FC I, II and III). With FC III (green excitation), only the globoids are visible (Fig. 5), while FC I and II may also permit simultaneous detection of other fluorescent structures such as autofluorescent blue, phenolic-enriched cell walls. The method is an empirical one, but offers striking sensitivity for detection of phytin globoids and is particularly suitable for high resolution analyses.

Storage Lipids

Commercial varieties of rapeseed contain abundant reserves of oil (approximately 40% by weight), primarily as hexane-soluble, neutral lipids (triglycerides) which may account for up to 95% of the total lipid in the seed. The majority of the storage lipid occurs in small (0.1-1.5 μm diameter), discrete, densely-packed droplets which surround the protein bodies and cell nucleus, as shown by electron microscopic studies (Hargin, 1977; Hofsten, 1974b).

Nile Blue A is an extremely sensitive and rapid fluorescent marker for rapeseed lipid reserves. Although the blue, aqueous solutions of the dye are not detectably fluorescent, a minor component (>3%) in most commercial Nile Blue A preparations rapidly partitions into neutral lipid-rich structures to produce intense yellow fluorescence which is detectable using FC II. The dye has been used previously to examine triglyceride-rich spherosomes in wheat (Hargin et al. 1980) and is equally applicable to hand-cut or modified GMA-embedded sections (Fig. 6). Because the Nile Blue A working solution is aqueous, there is no danger of lipid extraction during staining. The distribution of reserve lipids in hand-cut sections (Fig. 6) can be determined within 1-2 minutes. Intense staining occurs in virtually all embryonic cells, as well as in the aleurone layer. The aleurone layer adheres tightly to the outer seed coat structures and contributes to the high levels of lipid which are commonly found in experimental preparations of rapeseed hulls. Pretreatment with hexane completely extracts all stainable material from sections, which confirms that the dye has a very high sensitivity for neutral lipids. At higher magnifications (Fig. 7) individual oil droplets can be distinguished, and it is apparent that the distribution of stainable droplets mimics precisely that of supposed lipid droplets visualized in electron micrographs (Mills and Chong 1977; Hofsten 1974b). Emulsions of rapeseed oil and Nile Blue A produce fluorescent droplets which are microscopically indistinguishable in color from oil droplets observed in rapeseeded sections. Like several of the fluorescence procedures outlined in earlier sections, aqueous Nile Blue A also has considerable potential for use in rapid analysis of rapeseed products. For example, samples of rapeseed meal (Fig. 8) or other particulate products, can be examined immediately for residual lipid. Using this approach, it becomes a simple matter to estimate rapidly the extent of lipid removal during conventional crushing and solvent extraction procedures which are in commercial use. Cells which have not released their lipid reserves are identified clearly by their affinity for the Nile Blue A fluorochrome (Fig. 8). Modifications of this general technique for rapidly visualizing rapeseed (or other oilseed) lipids should also prove to be especially useful in expediting developments and/or improvements in experimental processing systems.

In this brief overview of techniques which are useful for the detection of major rapeseed components, it is apparent that the fluorescence microscope provides several advantages over conventional bright field optical methods. Most fluorescent markers offer improved sensitivity and contrast, the methods are generally rapid, and the epi-illuminating systems which are now available offer unprecedented resolution in relatively thick, hand-cut sections, as well as improved fluorescence intensity in thin, embedded sections. Fluorescence also permits localization of autofluorescent, phenolic-containing structures which are difficult to detect by other methods. We suggest that the fluorescence microscope is particularly well suited to a wide range of routine food analyses, primarily because of its exceptional flexibility and ease of application.

References


Discussion with Reviewers

J.M. Faubion: Which classes of phenolic residues do the authors feel could be localized by the fluorescence technique?

Authors: We have not yet attempted to identify specific autofluorescent phenolic compounds in the seed coat of rapeseed. However, the blue autofluorescence in cereal aleurone cell walls has been identified using fluorescence microscopy with a red filter in the excitation path. Similar techniques should prove useful in defining the classes of compounds which contribute to rapeseed autofluorescence.

J.M. Faubion: Since Hargin, et al. do not comment specifically on the minor component present in Nile Blue A, would the authors care to elaborate or speculate on its structure, possible isolation, etc.?

Authors: The oil-soluble fluorescent compound in Nile Blue A was first described by J.F. Thorpe 1907, J. Chem. Soc. 91: 324-336. It is readily isolated using organic solvents such as hexane or benzene.

B. Dronzek: Would it be possible to estimate the percentage of residual lipids in rapeseed meal using aqueous Nile Blue A?

Authors: An experienced microscopist could probably provide crude estimates of oil extraction efficiency on a routine basis by simple examination. Precise microscopic quantitation would require very additional, expensive instrumentation.

R.M. Saunders: Were the handcut sections from dry seeds?

Authors: Yes, they were from dry seeds.

R.M. Saunders: In the detection of carbohydrate material with periodate/Schiff's what happens to soluble sugars, e.g. sucrose, raffinose, etc? Are they 'washed out' by the aqueous nature of the detecting agent, or during presoaking of the seeds?

Authors: We assume that soluble sugars are removed by the aqueous fixative and/or dehydrating media during sample preparation.

D.W. Stanley: The enzyme myrosinase is of considerable interest in rapeseed due to its ability to break down glucosinolates to isothiocyanates which are potential goiterogens.

S.H. Yiu et al.
Have the authors attempted to identify any of these reaction products by fluorescence microscopy?
Authors: We have yet to identify a suitable fluorochrome for detecting myrosinase, glucosinolates or related reaction products. However, we are exploring potential bright-field reagents which appear to have some affinity for glucosinolate. Results of these studies will be published in the near future.

J.G. Vaughan: 'Morphologically discrete forms' - does this apply to vitamins?
Authors: Fluorescence methods have been developed to demonstrate that niacin is concentrated in distinct structures in cereal bran (Fulcher et al. 1981). We have not yet determined if similar vitamins-containing structures occur in rapeseed.

J.G. Vaughan: Which type of oil was used for mounting the sections?
Authors: A non-fluorescent immersion oil was used for mounting the sections for microscopic examinations.

J.G. Vaughan: The point about the Candle seed mucilage could be enlarged. It is sometimes stated that Candle is the result of a trispecific cross (napus x campestris x juncea). If this is the case, the Candle mucilage could be related to the juncea origin.
Authors: Confirmation of this suggestion will require a much broader varietal comparison than we have attempted in this study.

Authors: Thank you.

Editor: It will be useful to add micrographs showing the hot H2O extracted sections and the phytase-acriflavine work.
Authors: We agree. However, there are obvious limitations to inclusion and reproduction of too many color illustrations. We do have positive evidence supporting the claims made about the validity of these techniques in the text.

We shall be glad to send photographs to any interested reader upon request.
LIGHT MICROSCOPY PREPARATION TECHNIQUES FOR STARCH AND LIPID CONTAINING SNACK FOODS

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Abstract

Many processed foods lack the structural integrity associated with biological tissue so that the conventional methods of preparation and staining used in light microscopy may introduce misleading artifacts.

Taking as examples of starch-based processed foods, potato chips (UK potato crisps) and three distinct potato snack foods, methods for preparing and demonstrating the constituents present in cryosections of whole and masticated products are discussed. To show constituents in their true relative locations vapor staining and polarized light are used. Iodine vapor staining indicates the extent of starch gelatinisation in the dry snack and it is also used to show the structural changes that occur on mastication. Osmium tetroxide vapor colours the liquid fats and polarized light indicates the presence of crystalline fats and intact starch granules.

Introduction

All microscopy is subject to artifacts and for one to have confidence in the validity of the results it is important to recognize the artifacts associated with a chosen technique and to minimize or avoid them.

In light microscopy preparations must be sufficiently thin to transmit light and if the relative position of constituents is important then sections must be cut. For this the modern cryostat has many advantages (Bancroft, 1975). Fixed and unfixed material can be sectioned and embedding can often be avoided because many foods are moist and when frozen this moisture acts as the support needed for sectioning. Dry foods present a challenge because the unrestricted addition of water may greatly alter the very structure which is being examined. Fortunately dry foods do not have to be fully hydrated to permit sectioning. Provided that the sample size is small proprietary aqueous embedding media can be used to permeate the specimen sufficiently to give support without causing obvious swelling.

Staining presents the next opportunity to introduce artifacts. Aqueous iodine-potassium iodide solution is the classic method for demonstrating starch but many foods contain starch in a swollen gelatinized state and the addition of water further disrupts already fragile starch granules. Even intact starch granules may become detached from the slide during aqueous staining unless they happen to be firmly attached to the slide or surrounding material.

Although the iodide ion is necessary for iodine staining of starch because it forms an essential component of the starch-iodine complex, added iodide ions are not essential because iodine in the presence of water provides sufficient of these (Hollo and Szeitli, 1968). Clearly some moisture must be added to allow staining of the starch in otherwise dry preparations but the amount needed is small. This is the basis of iodine vapour staining (Little, 1957).

For the lipid staining the standard technique of using alcoholic solutions of oil soluble dyes such as Oil Red O or the more sensitive Sudan Black B are often satisfactory but they carry the risk of dissolving small droplets of lipid and of displacing low melting point lipids.

Aqueous osmium tetroxide avoids some of these problems but if the sections involved are sensitive to water then this method too is unsuitable. Osmium tetroxide is widely used as a fixative and users are aware of the hazardous nature of this toxic material which is exacerbated by its volatility. This volatility suggested the use of osmium tetroxide as a vapor stain for fat.

Complementary to vapor staining is the use of polarization

Key Words: Light microscopy, potato chips, fabricated snack foods, cryosections, gelatinized starch, lipids, iodine vapor staining, osmium tetroxide vapor staining, birefringence.
microscopy. Its value in observing starch based snack foods lies in its ability to demonstrate such ordered structures as intact starch granules, crystalline fats and cellulose derived from potato or cereal grain structures.

Materials and Methods

Reference materials: raw potato tissue sampled from the interior of a maincrop potato (variety, Majestic) commercial potato flakes prepared by a drum drying process.

The reference materials are included to illustrate the response of potato starch to cooking. Starch derived from raw potatoes and potato flakes are primary ingredients for snack manufacture.

Potato-based snack products: four distinctly different commercial snacks were examined:

1. A standard potato chip.
2. A stacking chip based on potato solids and cereal starch. This was similar in appearance to the potato chip but the moulding of the snack during manufacture produces uniformly saddleshaped items that stack together.
3. Product A: a puffed snack based on potato solids and cereal starch, initially cooked under pressure and extruded as a rounded pellet which is dried and later puffed by drying.
4. Product B: a puffed product based on potato starch and potato granules extrusion cooked and directly fried. Product B had the appearance of short lengths of spaghetti.

Section preparation.

Potato tissue was sectioned by hand using a single edged razor blade. All other materials were sectioned at 10μm using a Slee retracting microtome in a Bright cryostat at −20°C. The sections were collected on clean microscope slides. Prior to sectioning one of three procedures was used:

a) Potato flakes were broken into pieces about 2 mm in diameter and the pieces were mixed with Tissue-Tek OCT embedding medium and placed directly on the cryostat chuck (specimen holder).

b) Each snack product was broken into pieces 2-5 mm in diameter which were placed in a perforated stainless steel cassette. The cassette was placed in a beaker, covered in Tissue-Tek and evacuated slowly in a vacuum oven (without heat) to remove air trapped within the product's structure. When no more air bubbles were seen to escape the vacuum was slowly released. Individual fragments were then mounted on cryostat chucks.

c) Each snack product was masticated until thoroughly softened and a sample (ca 5 mm diameter) of the resulting pulp (masticate) was mounted on a cryostat chuck and covered with Tissue-Tek.

After procedures a), b) or c) the mounted specimens were quickly frozen in liquid nitrogen and then transferred to the cryostat. They were left to warm to cryostat temperature (−20°C) before sectioning.

Storage of sections prior to staining.

The slides holding the 10μm sections were stored at −20°C. This preserves the fat present in the sections and it ensures that when the slides are brought to ambient temperature a film of moisture will be present to aid iodine vapor staining.

Methods of staining

1. Demonstration of starch using iodine vapor.

The method is based on that used by Little (1957). An environment of saturated iodine vapor was produced in a small covered staining dish by placing in it a small watch glass containing finely ground elemental iodine, covering the dish and leaving for 5 min. (Caution! iodine is poisonous, do not inhale the vapor).

Individual slides were taken from the −20°C deep freeze cabinet and a film of moisture was allowed to form on each slide before quickly placing it in the covered iodine vapor dish. The slide was positioned so that the section could be observed from the outside. Optimum staining took about 1 min. Excess staining was avoided as this obliterated structural detail.

Mounting iodine stained sections.

A semi-permanent mounting medium based on Hinchman's mountant, (Hinchman, 1973) was used. The medium was prepared by warming 80 ml of a low viscosity corn syrup (Morsweet supplied by CPC United Kingdom) with 14 ml distilled water and 6 ml Lugol's iodine in 100 ml water. This mountant was found to preserve iodine staining for at least 6 weeks.

2. Demonstration of lipid material using osmium tetroxide vapor.

Osmium tetroxide is a toxic material its volatile nature making it especially hazardous. All the staining was done in a fume cupboard and gloves and goggles were worn when handling the 2% OsO₄ solution. A covered slide staining dish was used the base of which was covered with filter paper and two lengths of glass rod (5 mm diameter) were positioned on the base so that the slides would not touch the paper. A 5 ml vial of 2% OsO₄ solution was poured onto the filter paper, a slide was then positioned in the grooves of the staining dish and the lid was replaced. Sections were allowed to stain for 20 min.

Mounting osmium stained slides

Aquamount, a commercial aqueous mountant obtained from BDH Chemicals Ltd., Poole, Dorset, England, was used as a temporary mountant. The mounts were photographed immediately because the osmium stained oil was still mobile.

Polarization microscopy

Stained and unstained sections were examined for birefringence. Glycerol or Aquamount were used as mountants for unstained sections.

Results

Reference materials (Figs 1 and 2)

A comparison of these iodine stained sections shows the effect of heat processing on the starch present in potato cells. With the raw potato tissue (Fig. 1) many of the starch granules were lost during sectioning when individual cells were cut but the appearance of raw starch and the way it fills intact potato cells can be seen in two of the cells. The cryostat section of the potato flake shows how well the cooked potato cells retain their gelatinized starch during the processing of the flake. Although the starch loses birefringence during cooking the outlines of individual granules can still be seen.

Potato-based snacks (Figs 3-14)

Potato chip (Figs 3-6). In its whole state the potato chip was seen to be composed of mainly intact potato cells each filled with gelatinized starch. As with the potato flake, iodine vapor staining demonstrates individual swollen starch granules within each potato cell (Fig. 3). Osmium tetroxide vapor reveals the extent of fat penetration (Fig. 4), liquid fat is closely associated with cell walls and inter cellular spaces. Fig. 5 shows the presence of crystalline fat revealed by polarized light and the birefringence of the potato cell walls. The masticated chip sections when stained with iodine vapor show blocks of potato cells which have sheared away from one another during mastication. These cells still retain gelatinized starch which stains strongly
Figs 1 and 2. Reference materials stained with iodine:
Fig. 1 — potato tissue, intact cells (arrows) contain raw starch granules.
Fig. 2 — potato flake, cooked potato cells filled with gelatinized starch.

Figs 3-6. Potato chip:
Fig. 3 — iodine stained, gelatinized starch granules visible within cells.
Fig. 4 — OsO₄ stained, oil associated with cell walls (arrows) and intercellular oil (i).
Fig. 5 — unstained in polarized light, birefringent cell walls (arrows) and crystalline fat (f).
Fig. 6 — masticated chip, iodine stained, intact cells (i) starchy matrix (sm) and oil globules (arrows).
Figs 7-12. Potato-based snacks, iodine vapor stained:

Fig. 7 – stacking chip, starchy matrix (sm) entrains potato cells (p) and air cells (a).

Fig. 8 – stacking chip masticate viewed in polarized light, starchy matrix (sm) potato cells (p) and fat (f). Arrows show fat coating potato cells and dispersed in matrix.

Fig. 9 – product A starchy matrix of gelatinized granules (sm) contains cereal particles (cp) and air cells (a).

Fig. 10 – product A masticate, single starchy phase contains small voids (v) and some oil (arrows).

Fig. 11 – product B starchy matrix (sm) contains air cells (a) fat (f) and potato starch (arrows).

Fig. 12 – product B polarized light shows birefringent fat (f) and potato starch (arrows).
Potato-based snack foods

Cereal endosperm particles. The highly swollen starch granules of the matrix appear elongated and aligned end to end around the air spaces. On mastication the granular nature of the starch disappears (Fig. 10) the product forming a uniform starchy phase which contains small air pockets and some oil globules. Polarized light revealed no solid fat. In contrast, product B (Fig. 11) contains well defined starch granules and polarized light shows many of these to be intact (Fig. 12). Product B also contains potato cells which are easily seen in the iodine mount of the masticate (Fig. 13). Viewed in polarized light the iodine stained masticate reveals crystalline fat, intact starch granules and the outlines of potato cells can be seen (Fig. 14).

Concluding remarks

The methods described here to show the microstructure of potato snack foods could be applied to other starch and lipid containing foods. The value of iodine vapor staining is that it provides a sensitive test for assessing the extent of starch gelatinization without causing swelling. Osmium tetroxide vapor is especially useful for locating low melting point lipids when the use of lipid soluble colorants applied in solvent solutions would lead to movement and loss of the lipids.

However, the loss and movement of lipid material does not always accompany the use of oil soluble dyes. If a comparison of sections colored with an oil soluble dye such as Oil Red O or Sudan Black B shows similar results to sections stained with osmium tetroxide vapor then the oil colorant is to be preferred because of its greater safety in use.

The methods are intended to supplement rather than replace existing techniques e.g. in many instances a dilute solution of iodine in potassium iodide (the standard method for demonstrating starch) is quite satisfactory and the solution provides its own mountant which is convenient. However, if polarized light shows that the starch present has lost birefringence and is therefore susceptible to swelling in aqueous solutions, then the iodine vapor staining method and the Hinchman mountant are recommended.

Polarization microscopy provides an excellent means of discerning structure without introducing staining artifacts. This study shows it in use to demonstrate starch, cellulose and solid fats but it can also reveal other crystalline food constituents such as sucrose, lactose and calcium carbonate and oxalate.

Acknowledgements

Some of the practical work described in this tutorial paper was done by Miss Sandra F. Nelson and Mr. Michael H. Gamble as part of their individual student research projects. The author would like to record thanks to them and also to Leeds University Photography Service for the skilled preparation of the black and white photomicrographs from the original color transparencies.

References


Figs 13 and 14. Product B masticate:
Fig. 13 – iodine vapor stained, potato cells (PC) and starch granules (s) in starchy matrix (arrows).
Fig. 14 – iodine vapor stained viewed in polarized light birefringent starch (arrows) and crystalline fat (f) potato cells (PC) contents isotropic.

but the starchy contents of cells ruptured by the teeth are diluted by saliva and form a paler staining matrix in which droplets of oil as well as the potato cells can be seen.

Stacking chip. The iodine mount (Fig. 7) shows the microstructure of the dry product to consist of an aerated continuous pink staining matrix in which dark purplish blue stained potato cells are embedded. Gelatinized starch granules can just be seen in the mainly intact potato cells entrained by the completely gelatinized starchy matrix. The stacking chip contained more crystalline fat than the potato chip and this is well illustrated in the masticated sample (Fig. 8). This iodine mount shows intact potato cells against a background of starchy matrix which has now lost its aeration. Viewed in polarized light the crystalline nature of the fat present becomes apparent.

Puffed products A and B. Like the stacking chip both products have an aerated structure but iodine vapor staining and polarized light serve to show how different these products are. The iodine mount of product A (Fig. 9) shows it to be composed of a gelatinized starch matrix which entrains air and occasional cereal endosperm particles. The highly swollen starch granules

Photography Service for the skilled preparation of the black and white photomicrographs from the original color transparencies.
Discussion with Reviewers

Reviewer IV: What artifacts are possibly present due to tempering the sample at -20°C prior to cryosectioning?
Author: Tempering i.e. storage prolonged until an equilibrium of ice crystal formation is reached would almost certainly damage the specimen. Good cryostat technique involves very rapid freezing of a small specimen which is then brought to cryostat temperature and promptly sectioned. Liquid nitrogen (boiling point -190°C) provides rapid freezing but there would be considerable damage to both knife and specimen if an attempt were made to section the specimen at temperatures much below -30°C (Bancroft 1975). It is therefore important to allow the block to warm to sectioning temperature which takes about 10 minutes.

When material has been damaged by freezing (ice crystal artifact) the block is difficult to section and the sections show an uneven or torn appearance.

Reviewer IV: What artifacts may be induced by vacuum degassing prior to sectioning?
Author: A sudden application of reduced pressure could cause damage to fragile aerated samples. It is important to protect the specimen by enclosing it in a metal cassette and to evacuate slowly. A vacuum oven is useful because it carries a vacuum gauge so that the vacuum can be applied and later released in known easy stages.

The aim is to replace most of the air in the specimen with embedding medium and so ease subsequent sectioning but not all the air is removed. This can be demonstrated by very prolonged iodine vapor staining of the sections which show the extent of Tissue-Tek penetration which is colored yellow by the iodine. (With normal staining times the Tissue-Tek is barely visible.)

Reviewer IV: Why are air cells present in some structures after vacuum degassing?
Author: The air cells seen in the sections are an intrinsic feature of the fabricated snacks. These air cells can be seen if the products are broken and observed with a stereomicroscope using oblique illumination and focussing up and down.

Reviewer IV: What moisture content is present in these samples?
Author: The dry snacks contain only 1-2% of moisture, it is difficult to estimate the moisture content of the embedded material. The softness corresponds to a dry snack containing 10% additional moisture but the presence of the infiltrated Tissue-Tek makes comparison difficult.

Reviewer V: Why was crystalline osmium not used for more effective permeation of lipid components?
Author: The amount of osmium tetroxide required for vapor staining is quite small. The 5 ml ampoules of 2% osmium tetroxide which were used contain only 100 mg of osmium tetroxide. This amount of the solid would need careful weighing and would involve more handling of this very toxic material. It is therefore for reasons of safety that a solution was used. In laboratories used to handling osmium tetroxide the aqueous solution could of course be replaced by an equivalent amount of the crystalline chemical.

Reviewer V: Can you please discuss the implications of birefringence being induced in fat globules due to osmium complexing with lipids?
Author: In my experience with food lipids I have never observed osmium induced birefringence in fat globules. Fat globules often display birefringence but this is due to the unstained crystalline fat they contain and can be observed in unstained sections. Osmium staining will of course aid in the location of fat droplets and with partially crossed polars it will enhance the appearance of any birefringent material present by adding contrast to the image but this is not induced birefringence.

Reviewer V: Glass knives are routinely used for ultra low temperature microtoming. Have these been used on your samples? Does the level of magnification and/or resolution using the light microscope eliminate the problem of ice crystal damage that may be inherent in the preparation techniques now being used (with steel blade, ice crystal damage is certainly present at the ultra structural level)?
Author: Steel knives were used through this work. Provided that the specimen has been correctly frozen, ice artifacts are not a problem. This may be for the reasons you suggest but I believe that section thickness is an important factor. For much work in light microscopy 10μm sections are used, a thickness which is greatly in excess of the size of the ice crystals present. With ultra thin sections the way the knife deals with individual ice crystals is likely to be more critical.
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 (36) 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 microanalysis 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 permanganate 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 Pt/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, Fig. 3.

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 using Saltyav's microcapsule 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 dehydration 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. 5. 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% OsO$_4$ 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).
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

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**Fig. 8** Freeze-dried cryosection 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
freezing-etching, techniques which are discussed
by Buchheim elsewhere (12). Finally the cryo-
fixed 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 Savéus (64). After freeze drying
the sections are contrasted by exposure to
OsO₄ vapour. Fig. 8 shows the protein micro-
structure 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, Fig. 10. Submicellar-like particles have also been observed in thin-sectioned plastic embedded specimens such a structure is, although present, less clear,
Fig. 10. Submicellar-like particles have also been observed in thin-sectioned plastic
embedded samples of Camembert cheese (40) and in processed cheeses (29, 39, 42, 68). 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 dehydrator
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
are to be detected (50). 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-denaturated 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
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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 (18). 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 $\text{Os}_4\text{O}_4$, 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).

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 specimens 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 an electron from another shell. 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 electron 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 superposed. 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 $10^{-18}$ 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 Be 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.

**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. 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 leached away. O'Brien and Baumrucker 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. Insoluble material can be analysed as is demonstrated by the study of Blanc et al. 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 OsO_4 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 and embedding. Dehydration 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 sectioning, transmission electron microscopy the ideally frozen part of the sample, where no artefacts due to ice crystal formation have occurred, is either 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 much higher resolution which is also larger 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 specimen 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, a WD study 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.

Acknowledgements

I like to thank Dr. A. Boekestein, Technical and Physical Engineering Research Service, Wageningen, The Netherlands; the remaining micrographs were made by Mrs. P. Both, Netherlands Institute for Dairy Research, Ede, The Netherlands.

Fig. 8 was made in co-operation with Dr. P.M. Frederik and Dr. W.M. Busing, Department of Pathology, University of Limburg, Maastricht, The Netherlands. Figs. 15 and 16 were made by Mr. F. Thiel, Technical and Physical Engineering Research Service, Wageningen, The Netherlands; the remaining micrographs were made by Mrs. P. Both, Netherlands Institute for Dairy Research, Ede, The Netherlands.

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Discussion with Reviewers
M. Kalab: It is interesting to see casein
submicelles or the \( \kappa \)-casein-\( \beta \)-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 \( \text{OsO}_4 \), dehydrated with a graded series of
ethanol and impregnated with methacrylate
monomer. 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 denaturated whey
proteins are present.

D.E. Carpenter: Could the increased submicellar-
like particles in the freeze-fractured, sterile
and concentrated milk specimens be casein-whey
protein aggregates?
M.L. 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
sterilized, concentrated milks are not derived
from casein micelles?
Author: The submicellar-like particles most
probably are aggregates consisting of non-
icellar casein and denaturated 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 denaturated 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 ducupan or glycolmethacrylate
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 rotarily
shadowed at an angle of 10-25° with the
Milk and milk products

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.
The fluorescence microscope is one of the most sensitive instruments available for morphological and microchemical analysis of biological material, and especially of cereal grains. Recent innovations in illuminating systems, fluorescence chemistry, and specimen preparation have combined to provide significant improvements over conventional bright-field microscopy in both specificity and sensitivity. A variety of relatively specific fluorescent markers has been devised for routine and high resolution detection of all major cereal components. Several examples of useful fluorescent markers are described, including appropriate methods for specimen preparation, fluorescence analysis, and photography.

Introduction

Mature cereal grains contain a variety of nutritionally and industrially important constituents, including proteins, carbohydrates and lipids, as well as minor compounds such as phytin, vitamins, phenolic compounds (e.g. cinnamic acids, flavonoids, lignin), aromatic amines, nucleic acids, etc. All of these components are synthesized, packaged and stored in specific tissues, making the three major grain fractions (the bran, germ and starchy endosperm) chemically and morphologically distinct from each other. In short, the mature cereal grain is highly compartmented, and each morphological entity possesses distinct chemical characteristics.

In addition to obvious differences in chemistry and morphology among tissues in a single kernel, there are pronounced structural differences between equivalent tissues of different genera or cultivars, depending upon the genetic and/or environmental background of the parent plants. As these dissimilarities reflect chemical differences and thus determine the nutritional and processing characteristics of cereals, it is important to identify the extent of this variation to provide a sound basis for further improvements. Typically, tissue heterogeneity is best elucidated by applying simple microscopic techniques which define both the structural and chemical characteristics of grain components. Fluorescence microscopy is well suited to this task and, in combination with various specimen preparation procedures, provides considerable chemical information, simplicity, high resolution, and application to both intact and processed cereal grains. This overview outlines the advantages of the fluorescence microscope and illustrates methods for identification of specific cereal components.

The Fluorescence Microscope

The fluorescence microscope is one of the most sensitive chemical instruments available for cereal analysis, and it is also one of the simplest and most flexible. A suitable fluorescence microscope is a bright-field microscope fitted with a high intensity, broadband illuminator, and two filter systems for (a) providing excitation, or illumination, of the
Table 1. Spectral Characteristics of Fluorescence Filter Combinations

<table>
<thead>
<tr>
<th>Combination</th>
<th>Exciter Filter (transmission max., nm)</th>
<th>Barrier Filter (transmission max., nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC I</td>
<td>365</td>
<td>&gt;418</td>
</tr>
<tr>
<td>FC II</td>
<td>450-490</td>
<td>&gt;520</td>
</tr>
<tr>
<td>FC III</td>
<td>546</td>
<td>&gt;590</td>
</tr>
</tbody>
</table>

specimen (exciter filters), and (b) eliminating unwanted (excitation) illumination from the fluorescent image (barrier filters). Exciter filters commonly transmit in the ultraviolet, blue, or green regions of the spectrum and are inserted between the illuminator and specimen to maximize the excitation of the fluorescent compounds under investigation. Barrier filters are inserted between the specimen and the detector or ocular to remove all wavelengths, including excitation, shorter than that of the induced fluorescence. Thus the fluorescent image is viewed on a dark or black background and the high contrast provides the most important characteristic of the fluorescence microscope - sensitivity. As little as 10-10 moles of fluorescent material can be detected by microspectrofluorimetry (von Sengbusch and Thaer 1973). Spectral properties of filter combinations suitable for cereal analysis (designated FC I, II and III) are given in Table I. Others are available and may be used for special applications.

For most purposes, including cereal analysis, the fluorescence microscope should be equipped with an epi- (or incident-) illuminator such that the excitation illumination is reflected (by dichroic mirrors) through the microscope objective onto the upper surface of the specimen (conventional, sub-stage condensers illuminate the lower surface of the specimen). Fluorescence microscopy was introduced over seventy years ago, but it is the relatively recent development of epi-illuminators which has increased dramatically both the efficiency and application of fluorescence microscopy, for several reasons. First, because the epi-illuminator excites the top surface of the specimen, there are few problems with relatively thick sections, such as loss of intensity and resolution due to diffusion and absorption of fluorescence within the specimen. The latter has been a common problem with older fluorescence microscopes equipped with sub-stage condensers. Second, because the objective acts as the condenser, excitation occurs only in the area of the specimen being examined, and fading of fluorescence under high intensity illumination is restricted to the area of view (sub-stage condensers illuminate large areas of the specimen). Third, epi-illuminators dramatically increase the excitation intensity at the specimen surface with each increment in objective power. Therefore, fluorescence intensity, and hence sensitivity, increases as resolving power increases. This intensity improvement which is characteristic of epi-illuminators, combined with the availability of improved, high speed films now permits a satisfactory color or black and white photography of fluorescent objects in a few seconds, a fraction of the time previously required with sub-stage illuminators. All photographs included in this paper were recorded on Kodak High Speed Ektachrome (ASA 400).

Several conventional (bright-field) microscopic techniques also may be combined readily with epi-fluorescence systems for increased flexibility. Added information may be obtained by viewing a specimen simultaneously or sequentially using phase-contrast or polarizing optics in conjunction with fluorescence optics. Similarly, many fluorescent stains are also visible in normal bright-field illumination and it is often desirable to examine stained specimens with both fluorescence and bright-field optics. For example, in bright-field illumination, Congo Red imparts red coloration to a variety of cereal carbohydrates, but in the fluorescence mode only mixed linkage β-glucans are fluorescent. Most research microscopes are capable of combining fluorescence epi-illuminators with the usual range of conventional optical systems. The instrument used in this study is a Zeiss Universal microscope including a III RS epi-illuminating condenser with three standard filter systems (see Table I), and bright-field, polarizing and phase-contrast optics. Specialized techniques such as microspectrofluorimetry (Fulcher et al 1972), immunofluorescence (Fulcher and Holland 1971; Craig et al 1979; Gibbons 1980), and image analysis using fluorescence systems (Munck et al 1980) are beyond the scope of this discussion.

Sample Preparation
Preparative methods which are common to other light microscope analyses are generally also useful for fluorescence analysis. These include most fixation procedures, sectioning, dehydration or embedding equally to fluorescence analysis as they do to other forms of microscopy. For routine analysis of grains, it is often sufficient to simple cut relatively thin (10-20 μm) sections by hand using a clean razor blade and examine the sections directly for autofluorescence (see following section) or after the application of specific fluorescent dyes. Hand-cut sections offer the advantages of speed and minimal disruption or extraction of important constituents (e.g. lipids, vitamins) which might normally be lost to fixation, dehydration or embedding media. Some cereal grains (e.g. oats) are relatively soft and easily cut with a razor blade while others (e.g. hard wheats) are best softened before cutting by soaking in water at ice temperature. The epi-illuminating fluorescence condenser permits reasonable resolution of fluorescent structures in hand-cut sections. Similarly, ground or other processed...
Fluorescence Microscopy of Cereals

cereal samples (e.g. flours, concentrates) may also be readily examined for microchemical characteristics by fluorescence. Small amounts of powdered material should be mixed in one or two drops of fluorescence-free immersion oil under a cover glass for detection of primary fluorescence. For analysis after staining however, the nature of both the specimen and the fluorescent dye must determine the appropriate technique. Unlike conventional bright-field dyes, which are always deeply colored, some fluorochromes are not fluorescent until complexed with specific substrates. 8-Anilino-1-naphthalene sulfonic acid (ANS) for example, is not fluorescent in aqueous solution, but is intensely fluorescent when bound to hydrophobic sites on proteins (Weber and Laurence 1954; Gates and Oparka 1982). Thus, proteinaceous structures in flours or protein concentrates can be detected readily by mounting the sample directly in the aqueous fluorochrome solution under a cover glass; protein residues are intensely fluorescent and no extraneous fluorescence is evident. In other cases, excess fluorochrome must be washed from the sample after staining to minimize background fluorescence.

For high resolution fluorescence analysis, it is usually necessary to apply fixation and sectioning procedures which provide relatively thin sections (0.1-2.0 μm). Again, there are several techniques available, but the most useful of these employ glutaraldehyde fixation followed by glycol methacrylate embedding. Because mature grains are low in moisture (15%), and extremely dense, the most critical preparation step is fixation. It is normally desirable to prolong fixation times to allow adequate fixation and hydration of all tissues; apparently, tissues which are not sufficiently hydrated subsequently will not embed properly. A typical preparation schedule requires fixation of 1-2 mm thick slices of grain in 5% (w/v) glutaraldehyde in 0.1 m sodium phosphate buffer (pH 6.8-7.2) for 2-3 days on ice. Acrolein or formaldehyde may be included in, or substituted for, this fixative solution for special purposes. It is essential that heavy metals such as osmium or permanganate not be used; many heavy metals quench primary fluorescence in plant tissues, or interfere with the reaction of fluorescent dyes.

After fixation, cereal segments are dehydrated through methyl cellosolve, ethanol, n-propanol, and n-butanol and infiltrated with glycol methacrylate (GMA) monomer, and polymerized following the procedure of Feder and O'Brien (1968). Polymerized blocks containing tissue segments are cut to the desired thickness on glass knives using a Porter-Blum MT-1 ultramicrotome or similar instrument. Sections are then affixed to glass slides.

GMA is compatible with most aqueous staining solutions. The plastic is very hydrophilic and, unlike many epoxy-based embedding resins, does not interfere with staining reactions. The plastic also permits penetration by enzymes, allowing selective removal of cellular components by specific enzymes (Fulcher et al 1977).

Other methacrylate-based procedures may also be employed where it is necessary to minimize extraction of cellular components (especially lipids) during embedding. Hargin et al (1980) have adapted a "modified GMA" embedding procedure (Pease 1973) for cereal studies which is particularly useful for retaining neutral lipids (spherosomes) in situ. The fixed material is embedded directly in an aqueous mixture of urea/glutaraldehyde/glycol methacrylate and works well provided that water levels in the mixture remain relatively high (≥35%). As with the previous GMA procedure, the modified-GMA method readily produces sections of the requisite thickness (0.2-2 μm).

Fluorescence Microchemistry

(a) Autofluorescence

Many plant tissues contain a variety of substances which are naturally fluorescent (autofluorescent) under the appropriate excitation wavelengths and may be detected microscopically without further manipulations or staining procedures. Phenolic compounds are the most common autofluorescent materials in cereal grains and generally occur in cell walls of many tissues. For example, the numerous trichomes which occur on the surface of an oat grain are intensely autofluorescent using FC I (Fig. 1). The identity of the autofluorescent substance is unknown, but it is readily detectable under short wavelength excitation. The intense blue autofluorescence which is characteristic of the aleurone cell walls of wheat (Fig. 2) and all major cereal grains is produced by high concentrations of ferulic acid, a low molecular weight derivative of cinnamic acid (Fulcher et al 1972). Ferulate derivatives also occur in the seed coat (Fig. 2) and embryo, but not in significant quantities in the starchy endosperm of mature grains. Characteristically, fluorescent phenolic compounds undergo a significant shift in fluorescence emission spectra to longer wavelengths in alkaline conditions. Thus, exposure of sections to high pH (e.g. ammonia vapor) prior to examination often produces a dramatic color shift, as shown by ammonia-treated barley aleurone cells (Fig. 3). Based on autofluorescence characteristics alone, there is considerable potential for more precise identification of cereal components in situ by microspectrofluorimetry, and for monitoring concentrations of specific grain substances in industrial systems, such as flour milling.

(b) Induced (secondary) Fluorescence

Although many cereal compounds may be autofluorescent, most are not and must be converted to fluorescent compounds by chemical treatment. This may be accomplished by applying fluorescent dyes or stains (fluorochromes) or by inducing a specific reaction in sections in order to produce a fluorescent product. Specific fluorescence procedures have been developed at Ottawa Research Station for most major cereal components and several examples follow. Some of the procedures employ colored, bright-field stains such as Congo Red which are also intensely fluorescent under
appropriate excitation wavelengths. Others, such as Calcofluor and ANS, are colorless in visible light and are used only as fluorochromes. In some instances it has been necessary to adapt spot test or chromatographic spray reagents for histochemical use (e.g., for niacin and aromatic amine detection). Complete details of several of the following procedures have been described recently by Fulcher and Wong (1980), Hargin et al. (1980), Fulcher et al. (1981). Other procedures (e.g., for phytin and aromatic amines) represent new tests or improvements to earlier methods and complete details for their use are included.

(i) Phytin

Cereal phytin is a crystalline deposit of myo-inositol hexaphosphate, usually containing calcium, magnesium, and potassium. It occurs in the aleurone layer and scutellum of all cereals, and is the primary mineral reserve in the grain. In the past, phytin has been typically identified in grain sections by polarizing optics (the crystals are birefringent) or bright-field stains such as Toluidine Blue O (Jacobsen et al. 1971; Fulcher et al. 1981). These methods have been useful but both have limitations to their specificity. Many other grain components are birefringent in polarized light, and the typical red color of phytin after Toluidine Blue O staining is not specific to phytin.

In contrast, Acriflavine HCl provides improved specificity for high resolution fluorescence detection of phytin crystals. Sections are stained for 5-15 minutes in 0.1% (w/v) Acriflavine HCl in water adjusted to pH 3.1 with HCl. They are rinsed in ethanol until all traces of excess dye are removed, air-dried and mounted under a cover-glass in fluorescence-free immersion oil. Using this simple procedure, phytin crystals become fluorescent red when examined with any of the three excitation wavelengths described in Table I.

The apparent broad excitation spectrum of the phytin/dye complex demonstrates a significant advantage of fluorescence analysis. For example, short wavelength excitation (Wavelength I) permits simultaneous demonstration of autofluorescent protein bodies (yellow-orange) and cell wall ferulic acid residues (blue) as well as the stained phytin deposits (red) as shown in Fig. 4. At longer excitation wavelengths (green, FC III), only the phytin crystals are visible, and in very high contrast. Thus, fluorescence filter systems can be manipulated considerably to provide additional information. The chemical basis of the phytin/Acriflavine HCl interaction has not yet been determined.

(ii) Aromatic Amines and Niacin

It is a feature of fluorescence microscopy that a variety of fluorochromes or procedures may be available for detecting a particular compound or functional group. Therefore, a reagent may be selected to maximize color contrast with background autofluorescence, or to confirm the identity of specific components. For example, acidic dimethylaminobenzaldehyde (Ehrlich's reagent, DAB) was initially used to locate aromatic amines in cereal aleurone layers (Fulcher et al. 1981). The method is sensitive, but suffers from the disadvantage that the induced fluorescent reaction product is similar in its yellow color to occasional background protein autofluorescence. Substitution of a similar reagent (p-dimethylaminocinnamaldehyde, DAC) in the original procedure (see Fulcher et al. 1981 for details) produces a bright red fluorescent reaction product which is quite distinct from background fluorescence (Fig. 5). Cereal bran contains significant concentrations of α-aminophenol and its glycosides (Mason and Kodicek 1973; Fulcher et al. 1981) and the fluorescence colors of authentic α-aminophenol when reacted with DAB (yellow) or DAC (red) are visually indistinguishable from the colors induced in cereal aleurone layers by the same reagents. These reactions support the suggestion (Fulcher et al. 1981) that aminophenol is concentrated in the aleurone cells. Reaction products with DAB or DAC are not found in the scutellum.

Similarly, niacin residues can be demonstrated using a variety of methods based on detection of glutaraldehyde groups following cyanogen bromide (CNBr) cleavage of the niacin pyridine ring (Fulcher et al. 1979), the CNBr treatment, a yellow reaction product may be induced by reaction with p-aminobenzoic acid or an orange-red product (Fig. 6) with barbituric acid (Fulcher et al. 1981). The latter is preferable for distinguishing the niacin reserves from autofluorescent substances. The reaction is sensitive and readily demonstrates that niacin reserves are concentrated in specific sub-units of aleurone layer protein bodies (Fig. 6).

(iii) Storage Lipids

Triglycerides are stored in plants in spherosomes (oil droplets) bounded by a half-unit or monolayer membrane of proteins and diacylphospholipids (Yatsu 1972, Jelsma et al. 1977, Wanner and Theimer 1978). The aleurone layer and scutellum of most cereals are packed with lipid droplets and lower concentrations may also occur in the starchy endosperm. Nile Blue A is an excellent fluorochrome for demonstrating lipids (Wagner 1908, Thorpe 1907). However, in the presence of neutral lipids and short wavelength illumination, the red component becomes intensely fluorescent yellow and provides an excellent microscopic marker for cereal lipid reserves which are typically 60-70% triglyceride and other non-polar lipids (Sahasrabudhe 1979).

Complete details of the staining procedure have been described recently (Hargin et al. 1980). Briefly, hand-cut or modified GMA-embedded sections are stained with 0.01% (w/v) aqueous Nile Blue A. The sections are washed briefly, mounted in water and viewed immediately using fluorescence filter systems.
Fluorescence Microscopy of Cereals

FC II. Lipid reserves become intensely fluorescent yellow using this simple procedure and individual spherosomes can be resolved with little difficulty. Extraction of sections with hexane before staining confirms the identity of stainable reserves as neutral lipids. Figure 7 illustrates the distribution of neutral lipid reserves in a hand-cut section of an oat kernel. Lipid droplets can also be detected readily in flours by mixing a few drops of dye with a small amount of flour and viewing immediately.

In addition to the marked sensitivity of this dye in the fluorescence mode, Nile Blue A offers an additional advantage over other conventional lipid stains. Unlike the common Sudan dyes and Oil Red O for example, Nile Blue A is water-soluble and can be applied to specimens directly with little danger of lipid extraction during staining (presumably, the fluorescent derivative in Nile Blue A preparations partitions immediately into oil-rich reserves). In contrast, the common lipid dyes are applied in organic solvent/water mixtures. This procedure may extract a portion of the cellular lipids and invariably produces non-specific precipitates of dye on sections.

(iv) Cell Wall Carbohydrates

Two fluorochromes, Calcofluor White M2R New, and Congo Red are excellent microscopic markers for cereal endosperm cell walls. Calcofluor White is well known as a general stain for plant cell walls (Hughes and McCully 1975), and recent results have suggested that the fluorochrome exhibits a marked affinity for oat and barley β-(1-4)-(1-3)-α-glucans (Wood and Fulcher 1978). Congo Red also produces a fluorescent product with these carbohydrates and is particularly useful in detecting α-glucan residues in tissues where blue, autofluorescent phenolic compounds are indistinguishable from the fluorescence of Calcofluor-stained structures. Figure 8 illustrates Congo Red-stained components in the aleurone cell walls of oats (associated with blue, ferulic acid-rich areas), as well as in the thick sub-aleurone starchy endosperm cell walls. Calcofluor-stained material is shown in Figure 9.

Details of the application of these two fluorochromes have been described previously (Wood and Fulcher 1978; Fulcher and Wong 1980) and further discussion regarding the chemistry of their interaction with α-glucans and other polysaccharides is available (Wood, 1980). Similarly, the use of Aniline Blue as a potential marker for β-(1-3)-α-glucans has also been described (Fulcher et al 1977; Fulcher and Wong 1980). All of these dyes are simple to use and provide extremely high contrast for most applications.

Additional fluorescent markers are also available for other major cereal components and details of their use have been described earlier (Fulcher and Wong 1980). These include markers for protein such as 8-anilino-1-naphthalene sulfonic acid (ANS), Fluorescamine, Acid Fuchsin, and Orange G, and for starch, which is readily detected using the periodic acid/Schiff's (PAS) procedure. Other components for which fluorescent markers are now available include such diverse components as DNA, flavonoids, and lignin.

It is a feature of fluorescence microscopy that two or more fluorochromes may be applied to a single section or specimen to demonstrate the distribution of up to several substances simultaneously. Figure 9 for example, illustrates a section of oat kernel which has been stained sequentially with Calcofluor White M2R New (blue, for α-glucans), propidium iodide (red, for DNA), the PAS reaction (substituting Acriflavine HCl for Schiff's reagent to demonstrate starch grains, green), and ANS (blue-white, for protein bodies). Several other of the reagents described in this review can also be used simultaneously, or sequentially, providing that the staining reactions do not interfere. Because there are often two or more fluorochromes available to detect a particular cereal component, it is also possible to select dyes to provide maximum color contrast in sections stained with multiple fluorochromes (as in Fig. 9).

Concluding Remarks

The figures accompanying this review illustrate only a few of the many fluorescent markers which are available for cereal analysis. They have been selected to best demonstrate the advantages of the fluorescence microscope over conventional microscopic methods, namely improved sensitivity, contrast and, in most instances, chemical specificity. The methods are extremely simple, rapid and are readily applicable to many different types of preparations, especially whole grains and flours. It is anticipated that these developments should prove to be particularly adaptable for more precise evaluation of both the distribution and chemistry of seed components by image analysis and microspectrofluorimetry.

Literature Cited

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**Figure Captions**

Unless otherwise stated, all fluorescence photographs illustrate GMA-embedded sections of cereal grain tissues. Scale bars in μm. Abbreviations: a1 = aleurone layer; end = starchy endosperm; p = pericarp. Fluorescence filter combinations indicated as FC I or FC II (see Table I).

**Figure 1.** A sample of oat grain trichomes mounted in immersion oil showing intense autofluorescence. FC I.

**Figure 2.** A section of outer grain tissues of wheat showing blue ferulic acid autofluorescence in the aleurone cell walls (arrows). FC I.

**Figure 3.** A section of the outer tissues of barley kernel showing green autofluorescence in the aleurone cell walls after exposure to ammonia vapour. FC II.

**Figure 4.** A section of wheat aleurone cells showing red fluorescence in phytin granules (arrows) after staining with Acriflavine HCl. Blue autofluorescence is also visible in the cell walls (*) and aleurone protein bodies are yellow. FC I.

**Figure 5.** A hand-cut section of oat kernel stained with dimethylaminocinnamaldehyde (DAC) to show fluorescent red deposits of aromatic amines (arrows) in aleurone cells. FC II.

**Figure 6.** A section through wheat aleurone cells showing fluorescent orange nicain reserves (arrows) in the aleurone protein bodies after treatment with cyanogen bromide/barbituric acid. FC II.

**Figure 7.** A hand-cut section of oat grain demonstrating fluorescent yellow lipids reserves after staining with Nile Blue A. FC II.

**Figure 8.** A section of oat grain showing fluorescent red stained aleurone and endosperm cell wall components (arrows) after Congo Red staining. Ferulic acid residues are autofluorescent blue. FC I.

**Figure 9.** A section of oat grain stained using ANS, propidium iodide, Calcofluor White M2R New, and PAS treatments. Cell wall mixed-linkage β-glucans are blue, starch granules are green, nuclear DNA is red, and starchy endosperm protein bodies are blue-white. FC I.
Fluorescence Microscopy of Cereals
Discussion with Reviewers

J.M. Faubion: What are the limitations of the fluorescent technique (both in application and interpretation)? The initiated scientist may be inclined to plow ahead with this obviously powerful technique without an understanding of what it can and can't do.

A. Bridges: These techniques appear quite straightforward. Are there any cautions or warnings we should heed if we apply them to other plants or food materials?

Author: The primary limitations are chemical. It is essential that chemical studies parallel microscopic tests to establish the identity of seed components which autofluoresce or interact with fluorochromes. Once reasonable confidence in the identity of the appropriate compounds has been established, microscopic analyses may be conducted rapidly to provide considerable chemical information. In most of the examples illustrated in this overview, extensive chemical characterizations have been conducted over several years and are described in detail in the cited references. Extrapolation of staining results to biological systems other than cereals should be made only after similar studies are completed.

J.M. Faubion: For Figure 9, does the order of fluorochrome application affect the image quality or information content? In fact, could you elaborate on fluorochrome compatibility?

Author: Certainly the order of fluorochrome application will influence both image quality and information content. For Figure 9, for example, the PAS reaction was conducted first because the periodic acid adversely affects any staining reactions applied prior to the periodate. In addition to pH, one must also consider staining times, solubility of fluorochrome, and stability of tissue when devising multiple staining procedures. In general, staining specificity becomes less reliable as more treatments are used. Most microscopists would have little requirement to employ more than two stains simultaneously, and Figure 9 is used only to demonstrate that more than one component can be visualized in one section. It should also be noted that non-fluorescent dyes can be used in sequence with fluorescence methods to quench non-specific or background fluorescence and thus increase contrast.

A. Bridges: In some methods for fluorophore induction further sensitivity is achieved by using gaseous reactions and controlling the humidity. Would you expect a similar advantage for any of the procedures you mention? If so, have you tried using gaseous reactions for any of the examples you give?

Author: Gaseous reagents, if available, are advantageous in preventing or minimizing migration or extraction of cellular components during fluorophore development. In fact, the test for niacin employs cyanogen bromide vapor for this reason and the method is described fully elsewhere (Fulcher and Wong 1980).

A.A. Urquhart: You state that Nile Blue A is a specific fluorochrome for non-polar lipids. Is there any evidence that Nile Blue A stains a particular component of the non-polar lipids?

Author: Chromatographic evidence indicates that the dye is intensely fluorescent in association with triglycerides and less so with mono- and diglycerides.

A.A. Urquhart: Are there any fluorescent stains for polar lipids?

Author: Chromatographic studies also indicate that an additional component in Nile Blue A provides a fluorescent product with fatty acids and other polar lipids. However, in the conditions of the present study this interaction has not been exploited and does not interfere with spherosome detection. Studies are in progress to establish the chemical basis of this interaction.

A.A. Urquhart: Is there any evidence to suggest that treatments during processing of cereal grains might alter the specificity of reactivity of a stain for a particular compound? In other words, if, during processing, staining for a particular compound is no longer evident, can you be sure that processing has extracted that compound, or is there a possibility that processing has modified the compound so that the specific compound-stain reaction is eliminated?

Author: This is a dilemma which sooner or later confronts every microscopist and often it is by no means certain that lack of staining indicates absence of stainable substrate. Thus it is essential that food microscopy be fully familiar with the physical and chemical processes by which products are manufactured, and it is equally important that methods selected for microscopic preparation be understood and devised to provide minimal modification of components under investigation. All manipulations of raw materials will produce some degree of alteration during industrial processing or preparation for microscopy.
FREEZE-ETCH OF EMULSIFIED CAKE BATTERS DURING BAKING

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Abstract

Cryofixation, freeze-etch techniques were used to study the structure of cake batters made from a lean cake formulation before heating and after heating to temperatures up to 100-102°C. Batters were prepared without added emulsifiers and with saturated and unsaturated monoglycerides replacing 5 and 10% of the oil. Unsaturated monoglycerides were more effective than saturated monoglycerides in dispersing oil droplets through the batter. Saturated monoglycerides formed liquid crystals during baking. The temperature at which starch granules began to swell was slightly higher for saturated monoglyceride containing cakes. The batter matrix between starch granules was more clearly defined in unsaturated monoglyceride containing cakes.

Introduction

In a study of the effects of variations in the emulsive system on the structural and dynamic properties of cake batters (Cloke, 1981), it was found that the level and type of emulsifier affected heat transfer properties, water loss rates, air incorporation in batter, final cake structure as well as water loss rate upon reheating the cake. The integration of studies of food microstructure with other studies of changes at the molecular level as basis for understanding these macroscopic characteristics is described by Davis and Gordon (1982).

In this paper we report observations made with the freeze-etch technique of batter systems emulsified with saturated monoglycerides (SMG) and unsaturated monoglycerides (USMG).

The freeze-etch technique as applied to batter can be employed to assist in characterizing cake batter structure (Hsieh et al., 1981). It can be used to evaluate physical or structural events as they take place during heating of the batters. As such, it is useful in establishing interrelationships that exist among the components of complex macroemulsive systems. More specifically, changes in starch granule, oil, emulsifier and baking powder morphology as well as the distribution of batter components relative to one another can be observed. The observed physical changes are most likely the result of changes at the molecular level occurring in the system. However, the observations that are reported in this paper are made at the ultrastructural level and, as such, supplement other techniques such as differential scanning calorimetry (DSC) (Cloke, 1981) that document change at the molecular level. These techniques, in turn, can be used to explain the macroscopic changes related to final cake structure and dynamic characteristics related to water loss rates and heating profiles during baking.

Materials and Methods

Test batter formulation

The cake formulation is given in Table 1. Details of the modified two-stage mixing method can be found in Cloke (1981). Specifically, flour and baking powder were sifted together, and the oil (and emulsifier, if part of the
Table 1. Test Formula

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
<th>% (flour basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cake flour*</td>
<td>150.0</td>
<td>25.3</td>
</tr>
<tr>
<td>Baking powder b</td>
<td>7.1</td>
<td>1.0</td>
</tr>
<tr>
<td>(Sodium aluminum sulfate-phosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shortening</td>
<td>41.8</td>
<td>7.1</td>
</tr>
<tr>
<td>Corn oil c and d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoglyceride e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose solution</td>
<td>167.4</td>
<td>28.2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>107.0</td>
<td>18.2</td>
</tr>
<tr>
<td>Additional distilled water</td>
<td>19.5 f+ 100.0</td>
<td></td>
</tr>
</tbody>
</table>

aSoftasilk, General Mills
bCalumet, General Foods Corporation
cMazola, Best Foods
dDimodan PV, Grinsted Products: Mainly 85-90% glycerol monostearate and 10-15% glycerol monopalmitate (referred to as SMG). When SMG was used, it was heated to 60°C in 19.5 g water and allowed to cool to room temperature before being incorporated into the batter.
eDimodan 0, Grinsted Products: Mainly 70% glycerol monooleate (referred to as USMG). When USMG was used, it was heated to 60°C in 19.5 g oil and allowed to cool to room temperature before being incorporated into the batter.
f19.5 g water was added to batters containing oil alone or oil plus USMG, in order to maintain comparable volumes with oil plus SMG systems in which 19.5 g water was added with the SMG.

emulsifier:water:oil ratios and temperatures were used in the test bakings. Phase diagrams of SMG and USMG were used to select the combinations of water that were tested. The pretreatment for each emulsifier that gave the most acceptable cake in terms of volume, symmetry, cell structure and crust characteristics was selected for all further studies (Cloke, 1981).

Freeze-etch technique

The cryofixation freeze-etch procedure has been described by Hsieh et al. (1981). Sucrose, at the high concentration present in our batters, served as the cryoprotectant to prevent ice crystal formation. No additional cryoprotectants were used. Each sample of batter was placed in a specimen support disc, then frozen in liquid Freon 22 at -160°C. The specimens were fractured in a Balzers freeze fracture apparatus at -105°C. Etching took place for 2 min before the specimens were shadowed with platinum and carbon from a Balzers electron beam gun at an angle of 45°. The replicas were strengthened by the evaporation of carbon at a 90° angle. The bath components were dissolved from the replicas by flotation for 5–7 days in a solution containing 50% household chlorine bleach and 50% distilled water. The replicas were placed on formvar-coated slot grids and examined in a Philips 300 TEM microscope at an accelerating voltage of 60 kV.

Results and Discussion

As the first step in the study of the structure of emulsified batters, we examined the appearance of individual components of the system after dispersion in 42% sucrose, which is the concentration of sucrose present in the batters. The morphology of baking powder, starch from flour, and oil when using replicas in TEM, as shown in Hsieh et al. (1981), served as the basis for identifying these components in batters, the appearance and transformations of which is the subject of this paper.

Baking powder in the batters did not undergo an observable morphological change in the 40 to 102°C range. Although some of that originally present may have reacted or dissolved in the batter, recognizable structures were still present at 102°C.

Oil droplets also did not undergo an observable morphological change in the 40 to 102°C range and looked similar in appearance as those seen in Fig. 1. Fractures always occurred through the droplet.

Both the initial size distribution of the oil droplets (Fig. 1) and the changes in size distribution with heating (Fig. 2) were related to the emulsive system present in the batter. Unemulsified and SMG batters initially had a few small oil droplets and tended to form pools of oil (Fig. 1). After heating to 102°C, both the unemulsified and SMG batters showed irregularly shaped oil pools between amorphous starch granules (Figs. 2a, b). USMG batters initially showed both large and small droplets. This type of distribution of oil in the USMG batter persisted up to 102°C (Fig. 2c).

No structures assignable to USMG were
observed above room temperature. USMG was heated with oil to 60°C before it was added to the batter. It may have become associated with the oil prior to batter formation resulting in the fine dispersion of oil droplets observed in the batter.

SMG, in contrast to USMG, had distinct structural features which could be detected readily. Prior to batter preparation, SMG was dispersed in water and heated to 60°C. While some SMG may have formed mesophases, some appeared crystalline as can be seen in Fig. 3a for 10% SMG batter. Here we can see a surface fracture of an SMG particle. SMG appears to be made of layered sheets. At 89°C (Fig. 3b), a different crystal-like structure appears which is not associated with oil. According to Krog and Lauridsen (1976), a viscous isotropic and water mesophase could exist at this temperature. By 94°C (Fig. 3c), SMG partially encased oil droplets. Furthermore, SMG appears to be in lamellar or layered sheets similar to those present in the unheated batter (Fig. 3a). By the time the batter reached 100°C (Fig. 3d), SMG-oil interactions are once again observed with a variety of lamellar and crystalline forms present. It seems that SMG is present in several mesophase forms.

Thus, the oil/emulsifier/water system can be considered a three-phase system with a significant portion of the emulsifier present in mesophase structures. These micelles constitute a reserve from which emulsifier molecules can move to expanding interfaces to stabilize the interfaces. Furthermore, at some of the oil/water interfaces, the emulsifier is present in multilayers which would be expected to contribute to the stability of the system. The significance of presence and type mesophases in relation to starch transformations will be discussed following presentation of granule transformation.

Fig. 1. Freeze-etch micrograph of oil droplets from a 5% saturated batter (unheated).

Fig. 2. Freeze-etch micrographs showing oil distribution at the batter setting stage. (a) Unemulsified batter heated to 102°C (b) 10% saturated batter heated to 100°C (c) 10% unsaturated batter heated to 102°C
Fig. 3. Freeze-etch micrographs of the saturated emulsifier taken from 10% saturated monoglyceride batters at various stages of heating: (a) Unheated batter; (b), (c) and (d) heated to 89°, 94° and 100°C respectively.

Fig. 4. (a) Surface and (b) through fracture freeze-etch micrographs of starch granules from unheated, unemulsified batters.
As expected, the morphology of the starch granules changed during heating. Some of these changes, as observed by the cryofracture freeze-etch technique, were described in an earlier study of simplified model batter systems (Hsieh et al., 1981). However, there are some features of these morphological changes that occur at different times or in a slightly different manner when SMG or USMG are present in the batter. These differences may have implications for final cake structure as will be reported in a subsequent paper.

If we look at the surface of a starch granule in unheated batter (Fig. 4a), we observe a non-uniformity of surface structures that cannot be specifically attributed to protein, lipid, sucrose, or starch membranous material. Similar bumpy structures were also observed on starch in sucrose solution in the freeze-etch study of Hsieh et al. (1981). The cross-fracture for starch granules was difficult to obtain in unheated batter. In the example shown in Fig. 4b, the edges are clearly fractured with no evidence of a membrane on this granule. A fine granular appearance is present as described by Hsieh et al. (1981). All unheated batter formulations give similar results.

By 87°C to 91°C, the starch granules began differentiation from those in nonheated batters, and cross-fractures were frequent. By 94°C, almost all starch granules cross-fractured. By 102°C, there are no surface fractures of starch granules.

The temperature at which starch begins to change appearance (87-91°C) is altered by the presence of emulsifier. In Figs. 5 and 6, we show differences between unemulsified and some emulsified batters for surface and cross-fractured starch granules, respectively. We can see that...
Fig. 6. Freeze-etch micrographs of through fracture of starch granules from batters with different treatments heated to 87-91°C. (a) Unemulsified batter heated to 90°C (arrow points to rim); (b) 5% saturated batter heated to 91°C; (c) 10% saturated batter heated to 89°C; (d) 5% unsaturated batter heated to 87°C (also showing baking powder with sucrose solution); (e) 10% unsaturated batter heated to 89°C; (f) 5% saturated batter heated to 94°C.
the surface structure of granules in the unemulsified batter (Fig. 5a) is more smooth and layered than that of granules from the 5% and 10% SMG batter (Figs. 5b and 5c), respectively. The 5% USMG batter (Fig. 5d) shows a starch granule surface similar to the unemulsified one, but the 10% USMG batter (Fig. 5e) has an appearance midway between those in unemulsified batters and SMG batters. The cross-fractures through the starch granule in the unemulsified batter show distinct rims (arrow, Fig. 6a) that are present in the early stages of starch granule swelling. The reason for the formation of these rims is not known. It may be an initial stage in the plasticization of the granule with concomitant flow toward the edges. It is also possible that release of the soluble components is restrained by a membrane. However, the cross-fractured granules from unheated batters showed no evidence of such a membrane. Furthermore, in a study of granules,
utilizing ultra-low temperature microscopy, no evidence of a membrane was found (Davis and Gordon, 1978). The SMG batters contain starch granules that have few granule changes (Figs. 6b, c). The 5% USMG batter (Fig. 6d) contains starch granules that have a rim similar to those found in the unemulsified batter. The 10% USMG batter has starch granules (Fig. 6e) that have undergone some changes but not to the extent found in the unemulsified batter. Based on the criteria of presence of cross-fractures, granularity of interior, as revealed by cross-fractures, and development of a rim at the edge of the granule, the swelling order at about 90°C is: unemulsified > 5% USMG > 10% USMG > 5% or 10% SMG. Size of granule is not a useful criteria for this purpose because of the initial size variations and the uncertainty as to where the fracture occurs in the granule. It is not until 94°C that the SMG batter contains granules with rims (Fig. 6f) that closely approximate those seen at 90°C for the other systems. At the time of thermal setting of the batter (100-102°C), all starch granules have swollen and the internal appearance of granularity is different (Fig. 7). All batter preparations contain starch granules with nondescript boundaries, such as those seen for unemulsified batters in Fig. 7a. Batter with 5% SMG has some starch boundaries that were more clearly defined (Fig. 7a). Batter with 10% SMG (Fig. 7c) had less clearly defined starch boundaries. The boundaries between starch granules in USMG batters were also clearly defined, and there was less contact between granules than was the case in SMG batters. The oil appeared to be more finely dispersed throughout the matrix surrounding the granules. In Fig. 7e, the dispersion of the oil at the boundary of a group of overlapping starch granules is shown. Therefore, before the time of thermal setting, USMG disperses through the batter matrix, assuming that it remains closely associated with the oil. SMG, however, must undergo a series of phase changes (Figs. 2 and 3) before it becomes effective in encasing the oil later in the baking process (at about 95°C). Simultaneously, the initial stages of starch granule swelling are developed as shown in the sequences in Figs. 5 and 6. Eventually, all of the granules will undergo similar swelling at a higher temperature. Also, the matrix material between granules is more developed in the USMG batters at the time of swelling.

In interpreting these studies, it should be remembered that all experiments were carried out at the high sucrose concentration typical of cake batters. This has the advantage of studying the transformation as it occurs in batter. It has the further advantage in freeze-etch studies that the sucrose served as the cryoprotectant so that additional cryoprotectants were not required. Inhibition of swelling and elevation of gelatinization temperatures in model systems containing high sucrose concentrations was studied by Bean and Yamazaki (1978).

This, together with the starch:water ratios present in the batter, may account for some of the differences between starch transformation in the batters that we observed and those observed by others working with starch:monoglyceride:water systems in different concentration ranges.

The freeze-etch data presented here do not provide direct evidence of complexing of the emulsifiers with starch at the molecular level. DSC studies of this system (Cloke, 1981) indicated that the enthalpies of the starch transitions up to 120°C are the same, although the onset of the transition was delayed slightly at the 5% level of SMG. This result is supportive of the observations made with the freeze-etch technique which focuses on the changes at the ultrastructural level.

The critical role of water in starch granule transformation has been emphasized by many workers. Marchant and Blanshard (1978), in interpreting their small angle light scattering studies of starch granule transformation, felt that water must enter the starch granule by diffusion in order for molecular changes to occur. Van Lonkhuyzen and Blankestijn (1974, 1976) suggested that the monoglyceride micelle may form around the starch granule, for 1-5 Å to 10 Å from the surface of the granule. In this view, the monoglyceride does not enter the granule, although it may be tightly bound to it even at relatively low temperatures of 30°C. The monoglyceride would then act as a barrier to entry of water as suggested also by Eliasson et al. (1981).

If the monoglyceride does not enter the granule, enthalpies for starch transitions, as measured by DSC, and by implication the changes in molecular conformation of the starch components, would not be affected by the presence of monoglycerides as was, in fact, observed in our earlier study (Cloke, 1981) and by Eliasson et al. (1981) for potato starch.

From our freeze-etch studies of batters at room temperature (e.g., Fig. 3) it appears that much of the SMG remains in discrete particles at room temperature. From the distribution of these particles, it appears that close associations between SMG and the granules did not occur. While detailed layering of changes, starch swelling may be associated with the starch granule is beyond the resolution of our method and, therefore, cannot be ruled out. It would appear that SMG would be unable to interact with the starch granule to any great extent in the initial stage. USMG appears to affect the oil droplet distribution, but if it has an effect on the starch in the initially unheated batter, it cannot be seen at this stage of the batter development.

The freeze-etch studies of the transformation of SMG during heating reported here document the phase changes of SMG. If SMG requires mass transport of water into its crystalline structure during these phase changes, starch swelling may be delayed because of decreased availability of water for many of the chemical and physical transformations that precede or accompany swelling. In some of our earlier work with a variety of emulsifiers (Hsu et al., 1980) and in the investigation of the saturated and unsaturated monoglyceride batters (Cloke, 1981) in which water loss rates were monitored during baking, it was shown that, generally, the mass transfer of water out of the cake is delayed during this period of baking in
the presence of an emulsifier. Therefore, the water must be participating in some interactions even if it is blocked from the granule. Participation in crystal formation and oil micelle effects are possible explanations.

In our DSC studies (Cloke, 1981) SMG phase changes were observed to begin in the range of 57-59°C. Enthalpy changes were noted as oil and water were added, which suggest the formation of an SMG-water mesophase. Freeze-etch data show oil surrounded by SMG structures at 95°C, but similar structures were not found to surround starch granules. Other workers have attributed a surface adsorption role to the emulsifier. For example, Jongh (1961) stated that the adsorption of glycerol monostearate to the surface of the starch granule led to flocculation of previously stable starch suspensions. Thus, our freeze-etch studies do not confirm a surface adsorption role for the monoglycerides either in the initial or later stages of granule transformation.

The potential for complex formation between amylose and, to a lesser extent, amylopectin and lipids including monoglycerides has been recognized for many years. Many studies of complex formation were made with extracted amylose or amylopectin. When the granule is considered, either isolated from its original site or in the presence of other constituents found in its natural environment, as in the case of flour, the site at which complexing occurs (e.g., intra- or ex-granule), the stage in the heat-induced transitions at which it occurs, the consequences of complex formation on other aspects of the heat-induced transitions (most commonly - granule swelling, viscosity, extractability of monoglycerides (Krog, 1981)), as well as effects on overall product quality, have been the subject of much argument. Therefore, care must be taken to compare experiments that are measuring the same phase transitions or properties in starch gelatinization as Donovan (1977) has stated so clearly in the past.

The extent of complex formation with amylose is related to molecular structure of the lipids (Krog, 1981). Many studies have suggested that the differences between saturated and unsaturated monoglycerides are considered to be one basis for these differences. SMG, more than USMg, was observed in our studies to have an inhibitory effect on swelling of starch granules during heating.

For saturated monoglycerides, the nature of the mesophase has also been considered to play a role in the extent of gelatinization (Krog and Nybo-Jensen, 1970). It may be simply that the dispersion state influences the distribution of the monoglycerides throughout the system. Recently, however, Krog (1981) suggested that monoglycerides are adsorbed onto the granule surface in the α-crystal form and then are transformed to a liquid crystalline phase as the active form for complex formation. In his view, reduced granule swelling as a result of complex formation makes more water available for other reactions. As pointed out earlier, we saw no extensive adsorption of SMG on granule surfaces by freeze-etch technique. We suggest also that availability of water may be decreased by mass transport of water into the mesophas
Acknowledgement


References


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Discussion with Reviewers

J. Holme: The paper suggests one or two mechanisms by which decreases in water availability may result in less starch swelling and/or water loss from batter systems. Could you comment if and how EM might also be of value in demonstrating the role of other normal cake batter constituents, such as egg white and milk, in the thermal setting process of batter systems.

Authors: We have a study of this type in which dry nonfat milk solids additions are added to the lean formula described in this paper. We are measuring the macroscopic properties of the baked cake such as volume, color, cell structure; dynamic properties such as heat transfer and water loss rates and hope to correlate these with the microstructural changes observed with EM. We hope to characterize the changes in the microstructure of the components of the nonfat milk solids such as lactose and casein as well as changes in the components described in this paper such as oil, starch granules, and intergranular matrix. Studies, such as those of Buchheim, of the microstructure of milk products (Buchheim, W. A comparison of the microstructure of dried milk products by freeze-fracturing powder suspensions in nonaqueous media. Scanning Electron Microsc. 1981; III. 493-502), will aid in characterizing the initial structures before heating the batters.

P. S. Pescheck: Have you looked for starch-monoglyceride complexes by X-ray diffraction or tried to measure water activity in the presence of SMG or USMG?

Authors: We are planning to do X-ray diffraction studies in the next phase of our studies. We have determined the water activity of the cake "crumb" after baking. The values were in the range of 0.89 to 0.92 and did not appear to be related to the type of emulsive system being used.

P. S. Pescheck: I'm intrigued by the "bumpy" structure in Figure 4a. Have you seen them in systems which don't contain sucrose? What if you froze a suspension in oil—would you see them there too?
Freeze-etch Cake Batters

Authors: We have not prepared any samples without sucrose. The sucrose, in addition to its presence in the formulation, served as the cryoprotectant. Whether freezing in a suspension of oil without cryoprotectants would be successful would have to be investigated. Ultra-low temperature microscopy is an alternate approach.


Authors: We did not attempt any measures of solubility. If amylose solubilization occurred, the soluble amylose would become part of the intergranule matrix. In the freeze-etch studies, the matrix for USMG appeared to be more developed. It would be tempting to interpret this observation as support for increased solubilization of amylose in presence of USMG as compared to SMG, but further study is needed to support this interpretation.

SEM micrographs of starch granules from batters made with several different emulsifiers are given in Hsu et al. (1980). In these cases, a matrix is observed, but it is not fibrous appearing as shown by Hoover and Hadziyev. Ropey structures similar to those shown by Hoover and Hadziyev were observed occasionally in cakes baked in microwave ovens. They were observed in potato starch viewed at ultra-low temperatures (Davis and Gordon, 1978).

D. D. Christianson: Do you think that the SMG actually enters the granule and complexes with amorphous amylose inside the granule? Does this prevent solubilization? Because the thesis is hard to get at for the reader, I feel it would be valuable to include some backup DSC data, especially where oil-water-emulsifier play a key role in starch surface transition.

Authors: As we point out in the text, this question of the site of monoglyceride amylose interaction can only be answered indirectly in studies of this type. The freeze-etch technique can give information on the overall effects on the granule swelling. DSC studies give information on the enthalpies, and, as a result, conclusions about the conformational changes can be drawn. But neither technique unambiguously pinpoints the site of the interaction. Furthermore, it is our view that the question of amorphous amylose regions within the granule needs further investigation. One approach would be to form amylose-iodine complexes and then investigate the binding location of iodine with X-ray microanalysis.

Details of the DSC studies are given by Cloke (1981). As we point out in the text, in the batter formulation we did not see the endothermic peaks for complex formation reported by Kugimiya et al. (1980) and Kugimiya and Donovan (1981). However, almost all DSC studies show that the temperatures of onset of the peaks and enthalpies depend on starch:water ratios among other things. Thus, for our specific formulation we do not observe changes in enthalpy when emulsifiers, either USMG or SMG, are added although the temperatures of onset of peak change slightly.

R. G. Fulcher: Presumably, removal of samples from partially-cooked batters results in significant cooling and physical stress prior to freezing in liquid freon. Does this sampling procedure induce artifacts in the specimen?

Authors: Artifacts are always a possibility, but we tried to minimize the time between sampling and freezing, and to remove the sample with minimum handling. The batter remains quite fluid up to 102°C, so that it is easily sampled. In the 100-102°C range, structure is just beginning to form so, again, the crumb is not subjected to cutting stress.
ASPECTS OF SAMPLE PREPARATION FOR FREEZE-FRACTURE/FREEZE-ETCH STUDIES OF PROTEINS AND LIPIDS IN FOOD SYSTEMS. A REVIEW

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Abstract

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

Introduction

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

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

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

1. Chemical fixation

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

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

In the following text a few personal experiences are summarized in order to draw attention to possible problems with chemical fixation. First of all it should be emphasized that the cross-linking effect of fixatives such as glutaraldehyde will induce more serious changes in sols than in systems with an already organized, i.e. cross-linked structure (gel). For example, the effect of glutaraldehyde on a protein solution might be the formation of artificial aggregates whereas the network structure of a system like Cottage cheese is probably further stabilized. We have observed such a distinct formation of aggregates in a 3.5% solution of β-casein (9). Without fixation a more or less uniform distribution of the individual protein particles (10-20 nm in diameter) was found (Fig. 1), whereas a 1-hour fixation with 1% glutaraldehyde produced a pronounced degree of particle aggregation (Fig. 2) which was accompanied by a visible increase in the turbidity of the solution. When glutaraldehyde acts on protein sols of higher concentrations such as a 12% soy protein sol, it immediately transforms it into a solid gel and also induces a certain degree of protein aggregation (23).

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

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

Finally, experimental conditions will shortly be described which require chemical prefixation prior to any further preparatory steps. As will be further outlined in Sections 3, 4, and 6, the freeze-fracture/freeze-etch methodology enables us to make structural details visible in different
ways by modifying the aqueous phase of the specimen. For all deep-etching experiments (see Section 6), we need pure distilled water (or at least a highly diluted aqueous phase) in order to make the true outer surface of the aggregates visible without risk of contamination from dissolved material. Since proteins are sensitive to such drastic environmental changes (e.g., casein micelles disintegrate into casein submicelles when the original milk serum is replaced by distilled water), their structural organization must be chemically fixed in advance. Furthermore, it will be shown that it can be advantageous to freeze-fracture and freeze-etch an originally aqueous system in a non-aqueous medium such as dioxane or ethanol (12, 27) in order to modify the fracturing characteristics or to improve the cryofixation. Of course, such experimental conditions require similar chemical pretreatments (glutaraldehyde and/or osmium tetroxide fixation as in preparing specimens for thin-sectioning) in order to stabilize the system during the dehydration step.

2. Cryoprotective pretreatment

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

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

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

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

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

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

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

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

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

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

3. Cryofixation of aqueous systems

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

3.1. The standard freezing technique

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

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

Figures 3 and 4 demonstrate further that occasionally some particulate constituents may remain within the ice phase. A comparison of the average ice crystal size in both samples
Comparable structures have been found in gelatin gels (7, 64), gels of α- and β-casein (8), vicla fava protein gels (48), and potato starch gels (63). In all these studies the authors have considered the resulting network structure as the real gel structure instead of representing freezing artefacts.

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

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

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

Systematic studies on the freezing properties of systems having a tower water content but a continuous water phase, e.g. oil-in-water dispersions (emulsions), have not yet been made. In contrast, dispersions (emulsions) of the water-in-oil type such as margarine and butter have been studied by freeze-fracturing/freeze-etching (14, 45). The finely dispersed aqueous phase with droplet diameters of up to 10 μm and more exhibited a very low degree of ice crystal formation as can be seen in Fig. 5 (margarine, freeze-fractured) and in Fig. 6 (butter, freeze-etched). The reason for the absence of larger ice crystals in these droplets cannot be due to a higher cooling rate because of the low thermal conductivity of the fat phase. Perhaps the greater degree of supercooling of such small volumes is responsible for this effect. Starting from this observation, we developed a special freezing technique, the so-called oil emulsion freezing technique, which will be discussed in greater detail in Section 3.2. However, the results obtained with margarine and butter, the water content of which is approximately 15% (v/v), do not allow further conclusions to be made for such systems with higher water contents.

3.2. Ultrarapid freezing techniques

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

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

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

The normal method of handling the frozen droplets is to replace the propane by another suitable cryogenic liquid, e.g. n-butylbenzene (melting point 185 K), at temperatures well below the recrystallization temperature (e.g. 188 K) and finally to freeze 1-2 mm³ of a highly concentrated suspension of the frozen droplets in this second liquid mounted on normal freeze-fracture specimen holders by immersion into liquid nitrogen.

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

We have used this technique for studying the monomer-polymer equilibrium of β-casein (16) and the size and molecular weight of α-lactalbumin and β-lactoglobulin (51).
Should this technique be applied to systems containing aggregates which react sensitively to shear forces, attention should be paid to possible structural changes (deformations) during droplet formation. In protein-containing systems such as skim milk we observed an adsorption of casein micelles at the droplet surface. This must be taken into account, especially for quantitative measurements (Fig. 13). Shalaby and Severs (47) recently compared the cooling rates achieved within such a sandwich specimen when frozen in a propane jet device and when alternatively plunged by hand into liquid propane, and found no substantial differences.

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

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

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

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

4. Cryofixation of non-aqueous media

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

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

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

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

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

5. Freeze-fracturing

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

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

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

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

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

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

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

The freeze-fracturing characteristics of protein aggregates in organic solvents such as toluene and dioxane differ markedly from those in aqueous media (12), as most aggregates are no longer cross-fractured but surface-fractured. This fundamental difference is demonstrated in Fig. 15, 16, and 17, presenting micrographs of the same type of sample (rennet-coagulated casein micelles fixed with glutaraldehyde). Recently Kalab (27) freeze-fractured a (chemically fixed) yoghurt sample in ethanol and subsequently dried it for scanning electron microscopic studies. He observed similar differences between the fracturing of the protein aggregates in an aqueous and in the...
Fig. 18, 19, 20, and 21. Fat globules in fresh, not cooled milk exhibit 4 major fracture types. (I): cross-fractured globules, in which the fat phase (F) is exposed. Occasionally layered structures (L) appear, which are still different from really crystallized fat. The fat globule membrane (M) is exposed only locally (Fig. 18). (II): exposure of the outermost layer of the fat core due to fractures between the inner surface of the fat globule membrane and the fat core. Fig. 19 shows the smooth appearance of this surface which is only interrupted in a limited area (1), where the fracture went deeper into the fat phase. (III): The complementary view after such a fracture reveals the inner surface of the fat globule membrane (iM) to which small portions of fat (F) occasionally adhere (Fig. 20). (IV): Only rarely do fractures between the outer surface of the fat globule membrane (oM) and the serum occur. In Fig. 21 the fat globule membrane has been partially removed, exposing the outermost layer of the fat core (F). In Fig. 21 ice crystallization occurred despite glycerol cryoprotection. This is probably due to inadequate mixing with the milk. (IC: ice crystals; EP: eutectic phase). Bars, 0.5 μm.
The freeze-fracturing characteristics of lipid phases and lipid aggregates are determined mainly by the amounts and spatial distribution of molecularly well-ordered ('liquid crystalline') and non-ordered (liquid) lipids, and also by the size of aggregates and the type of interfacial layers. Milk and dairy products are well suited to demonstrate the main differences in appearance. On the one hand, the milk fat is a complex mixture of different high-melting fat fractions which exhibit peculiar crystallization and melting phenomena, and on the other hand, the state of dispersion of the fat phase and the type of interfacial layers undergo severe changes during processing. Within the lactating cells of the mammary gland, fat droplets (several micrometers in diameter) are formed. During secretion they are enveloped in (apical) plasma membrane, the so-called milk fat globule membrane (MFGM). This interfacial layer is responsible for the high emulsion stability of the fat phase in fresh milk (39). Although the milk fat contains fat fractions with melting points higher than body temperature, the fat is totally liquid when secreted and crystallization processes do not start unless fresh milk is cooled below 293 K. Freeze-fracturing of fat globules in fresh uncooled milk results in the following fracture characteristics: They are either cross-fractured at the same level as the surrounding aqueous phase or are fractured along the periphery. Cross-fractured fat globules (Fig. 18) exhibit the typical fine structure of non-crystallized fat. Although it largely appears amorphous, smaller areas with a very characteristic layered structure can frequently be found. According to our experience, such layers seem to represent a (non-crystallized) state where triglyceride molecules of similar length are somewhat loosely packed together in monolayers (15). These layered structures occur predominantly in a temperature range between body temperature and the temperatures where true crystallization starts within the fat globules (230-280K). There are no indications so far that these structures are smeared during cryofixation. Cross-fractured fat globules only occasionally reveal small portions of the MFGM (see Fig. 18). Cross-fractured areas of the MFGM very often show numerous small (approx. 10 nm wide) particles which should largely represent membrane proteins (enzymes, glycoproteins etc.) because up to 60% of the dry weight of the MFGM is protein (39). Peripheral fractures of fat globules in (uncooled!) fresh milk occur predominantly at the inner surface of the MFGM, i.e. between the MFGM and the fat core, and only very rarely at the outer surface, i.e. between the MFGM and the milk serum. An internal cleavage of the MFGM as in biomembranes (55, 57, 61) almost never occurs in expressed milk. A bilayer structure has only occasionally been found in a narrow zone in cross-fractured MFGMs (6).

To demonstrate the three possible cases of surface fracture of fat globules in fresh milk, compare Fig. 19, 20, and 21. Fig. 19 shows the smooth outer surface of the fat core, interrupted in a limited area to reveal underlying monolayers of fat. The complementary event is shown in Fig. 20, where the inner surface of the MFGM, with fat adhering to a limited area, is exposed. A view of the true outer surface of the MFGM is seen in Fig. 21. It can be seen that certain parts of this MFGM must have been removed with the complementary part of this fracture. An explanation for the preferential fracturing between the inner surface of the MFGM and the (previously) liquid fat core could be a much stronger interaction of the phospholipid molecules (and membrane proteins?) with the milk serum than with the fat. The reason for the absence of internal fractures of the MFGM which one would expect if the original phospholipid bilayer structure of the plasma membrane still existed, is not yet fully understood. Possibly a reorganization of membrane constituents after secretion takes place due to the presence of (apolar) triglycerides at the inner surface of the MFGM. This could, of course, substantially alter the fracturing characteristics. Alternatively, freeze-fracture studies on the secretion mechanism of fat globules indicate a 'clearing of membrane particles' on those parts of the plasma membrane which are directly involved in the secretory event (40).

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

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

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

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

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

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

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

6. Freeze-etching

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

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

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

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

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

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

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

than 10 mM) should be used, in order to avoid artefacts caused by a deposition of dissolved material (33). Although aqueous systems generally have to be cryofixed by ultrarapid freezing techniques to obtain suitable conditions for etching, freezing artefacts need not always interfere with a deep etch. This has been demonstrated by Stae-helin (57) who used the standard freezing technique for erythrocyte membrane ghosts in a dilute buffer. After freeze-etching, he obtained views of large areas of outer membrane surfaces which are normally not exposed by freeze-fracturing. Similarly favorable etching conditions have been found for the fat phase of an emulsion, the serum phase of which had been replaced previously by distilled water or a dilute buffer (22, 41). It is striking that, although cryofixation by the standard freezing technique has been used, freezing artefacts have hardly been detectable in systems of so-called washed cream (30-50% fat), i.e., the fat phase of milk after repeated centrifugation and redispersion in distilled water. Large areas of the true outer surfaces of fat globules, i.e., the fat globule membrane, were obtained by deep etching of such systems (See Fig. 29). Of course, the exchange of the milk serum for distilled water could eventually result in structural changes of such interfacial layers unless this structure is previously cross-linked by chemical fixation. If original cream is cryofixed and freeze-etched, the outer surface of the fat globule membrane becomes visible only in a very narrow zone (6, 22).

For studying the size and shape of hydrated protein molecules by freeze-etching, ultrarapid freezing techniques such as the spray-freezing technique have to be applied (2-5). We have used this method for studying the temperature-dependent aggregation of β-casein (16) and for visualizing α-lactalbumin and β-lactoglobulin molecules (50). These globular proteins are hardly detectable in freeze-fractured preparations (in contrast to the randomly coil ed caseins) but exhibit a diameter of approximately 15 nm in a freeze-etched preparation (see Fig. 35). The differences in size between freeze-fractured and freeze-etched protein particles have to be ascribed to the fact that during freeze-fracturing the particles are severely damaged, i.e., mostly torn apart, whereas they are completely set free during the etching process. Furthermore, the fine network of gel systems can be made visible, because freeze-fracturing alone cannot provide the necessary information in the third dimension. The application of ultrarapid freezing methods is essential for such highly hydrated systems (17, 23). As examples, the heat-coagulated soy protein gel (Fig. 9-11) and the sol-gel transformation of gelatin (Fig. 12 and 13) can be taken.

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

In this context it should be mentioned that Hood and Allen (24) freeze-etched carrageenan-milk gels in the presence of 20% glycerol, which was not high enough to prevent freezing artefacts but which enabled the occasional detection of fibrils which were supposed to represent the gel network. Generally, various types of etching (and condensation) artefacts can occur in glycerinated samples (9, 10, 56). Fig. 30, 31, and 32 illustrate that pits of varying sizes and shapes can appear as the result of random or controlled etching. It is also noteworthy to realize that protein molecules which have been deformed during freeze-fracturing are able to partially restore their original structure during freeze-etching. This has been demonstrated with catalase crystals (30). Very dense protein matrices as they are present in natural hard cheese varieties or in process cheese exhibit a considerably finer particulate structure after freeze-etching, which results in a slight sublimation of ice, as compared with

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Fig. 30, 31, and 32. Various types of artefactual etching patterns may occur in glycerinated aqueous phases. Fig. 30 shows a solution of β-casein (see also Fig. 1) where a short uncontrolled etching occurred at 163 K and produced small depressions (D) of approx. 50 nm in diameter. P: protein particles. Fig. 31 (an emulsion of butterfat in whey) and Fig. 32 (whole milk) are of freeze-etched preparations (173 K, 1 min) of cryoprotected (33% glycerol) systems, which exhibit an uneven sublimation of ice. F: parts of cross-fractured fat globules. Bars, 0.5 μm.

freeze-fractured preparations (Fig. 33 and 34). However, the structural differences between different types of cheese are easier to detect in unetched preparations, as already mentioned in Section 5. In earlier freeze-etch studies of Cheddar, Cheshire, and Gouda cheese (21), differences between the protein matrices were reported but these specimens had been cryoprotected with glycerol whereas those shown in Fig. 33 and 34 were not. Finally it should be mentioned that media other than water allow a controlled sublimation. It is demonstrated in Fig. 17 that dioxane sublimes under conditions which are somewhat similar to those for water.

7. Replication

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

Although unidirectional shadowing is normally most suitable for replication, the adaptation of rotary shadowing to freeze-fracturing/freeze-etching has resulted in an improved presentation of subunit structures of protein particles on biomembranes (31). Recently we applied this technique to molecular aggregates (submicelles) of casein which had been spray-freeze-etched in a dilute buffer in order to study the true size of these particles. Fig. 35 shows unidirectionally shadowed β-lactoglobulin molecules, and in Fig. 36 rotary-shadowed casein submicelles are shown. The high contrast at the periphery of the rotary-shadowed particles allows more precise measurements of size and shape to be made than on unidirectionally shadowed preparations. However, the interpretation of rotary-shadowed specimens is impaired by the observation that the appearance of small particles such as ferritin molecules varies dramatically depending on the shadowing angle and the thickness of the metal film (38). The apparent size of unidirectionally shadowed protein particles in freeze-etched preparations, measured perpendicularly to the direction of shadowing, has to be reduced by approximately twice the thickness of the deposited heavy metal film (generally 1.5-2 nm) in order to obtain the true diameter of the hydrated particle (52). Although the quality of the shadowing layer largely determines the resolution of the replica, the additionally deposited layer of pure carbon (10-30 nm) can result in peculiar contrast
Fig. 33 and 34. Two views of the protein matrix of a non-cryoprotected process cheese sample, one obtained after freeze-fracturing (Fig. 33) and the other view obtained after freeze-etching at 183 K for 1 min (Fig. 34). Protein particles are easier to identify in unetched preparations. Bars, 0.5 μm.

characteristics at the periphery of free particles (53).

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

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

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References


Discussion with Reviewers

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

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

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

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

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

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

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

Author: No. I am not aware of any application of this technique for gels elsewhere.
Plastic fats consist of a three-dimensional network structure of crystals in which liquid oil is trapped. This crystal network is held together by weak attractive forces, the nature of which is not definitely established. Crystal size is dependent on temperature history and is subject to polymorphic transitions which greatly affect the microstructure of the system. The microstructure of fats has been investigated by using polarized light microscopy, electron microscopy and X-ray diffraction analysis. Recently, a permeametric method has been developed which enables the determination of the specific surface area of the crystals in a fat. This method is a useful complement of the microscopic techniques. Scanning electron microscopy has not been widely used in studying fat crystal structures. The use of microscopy in the study of microstructure of emulsions presents even greater problems than in the fat field. Emulsifiers may form liquid crystalline mesophases which may be studied by polarized light microscopy and X-ray diffraction analysis.

It is becoming increasingly apparent that our understanding of what is now being described as the "functional properties" of many foods is dependent on a knowledge of their fine structure. This can be expressed in the following inter-relationship:

chemical composition + physical structure = physical properties

This indicates that microstructure is dependent on the chemical components of the food, and in turn, the nature of the microstructure determines the physical properties which include a number of functional properties. Fats and fat containing foods are no exceptions to this rule and much research has been devoted to the microstructure of fats. There are a number of factors which make these products unusual and the study of their microstructure difficult. Probably the most important of these is the influence of temperature. Not only is the microstructure of fats temperature dependent, it also depends on temperature history so that two samples of a fat examined at the same temperature may have different properties depending on their temperature history. The physical structure of fats may also change with time at constant temperature because of polymorphic transformations. These factors make it necessary for research on fats and fatty foods to be conducted in or with temperature controlled facilities. The study of emulsions also involves unusual problems since the membrane structures around emulsified droplets and their properties require specialized techniques for their observation and measurement. A good general source of information is the book "Food Microscopy" (Vaughan, 1979).
Fats differ from oils in that they contain solid triglycerides at room temperature. This mixture of solid and liquid components exhibits the property of plasticity. Triglycerides are long chain molecules with molecular weights in the neighborhood of 900. The crystallization behavior of such compounds responds to the normal conditions of nucleation and crystal growth (Figure 1), and these factors are determined by the degree of supercooling. If the extent of supercooling is low, only few nuclei will form and crystals will grow to a relatively large size. If heat is rapidly removed, i.e., high level of supercooling, many nuclei will form and many small crystals will be formed. The situation is complicated by the phenomenon of mixed crystal formation (also called solid solutions). Fats contain many different glycerides which closely resemble each other but have slightly different melting points. Rapid cooling (high supercooling) will result in inclusion of different types of triglycerides in the crystal lattice. The result is higher solid fat content at low temperature. Tempering of such rapidly cooled fats at temperatures below the melting point will lead to recrystallization and reduction in solid fat content. An example taken from the work of Hertens and deMan (1972) illustrates this phenomenon (Table 1).

Fats are subject to some additional phenomena when crystallization occurs. Long chain compounds including the triglycerides show polymorphism, i.e., they can occur in several crystal modifications. These different crystal forms are distinguishable by X-ray diffraction, by infra-red spectrophotometry, by melting point determination and by differential thermal analysis. It is generally agreed that fats occur in three major polymorphic forms, named alpha, beta-prime and beta in order of increasing stability (Lutton, 1972). The packing of the triglyceride molecules in the crystal lattice determines the spacings between adjoining molecules. The cross-sectional structures determine the short spacings which can be determined by X-ray diffraction. The arrangement of long chain compounds in a cross-sectional view of the crystal lattice has been given by Lutton (1950) and is represented in Figure 2. The alpha form is hexagonal and is the least organized of the three forms. It has a low density structure with a cross section of about 0.2 nm and the chains are packed in an untitled or perpendicular fashion. This form is usually obtained on rapid cooling of the melt. The beta-prime structure is orthorhombic and is in a tilted chain

Table 1. Solid fat content of a margarine oil and a frying fat with and without tempering at 25 °C for 30 min. Initial cooling at 0 °C for 60 min.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>10°</th>
<th>20°</th>
<th>30°</th>
<th>40°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Margarine</td>
<td>Tempered</td>
<td>37.6</td>
<td>24.2</td>
<td>11.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Non tempered</td>
<td>47.3</td>
<td>29.8</td>
<td>11.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Frying fat</td>
<td>Tempered</td>
<td>63.7</td>
<td>45.7</td>
<td>25.8</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Non tempered</td>
<td>75.8</td>
<td>55.1</td>
<td>26.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Fig. 1. Schematic representation of the rate of nucleation and crystal growth as influenced by supercooling. A = start of nucleation, FP = freezing point.
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Fig. 2. Cross-sectional structures of long chain compounds. (Source: E.S. Lutton, 1972. Reproduced with permission).

Row 1
Alpha: 1-0-0

Row 2
Beta Prime: 2-0-0

Row 3
Beta: 3-0-0

arrangement. This is the more common form for many natural fats. It is a more closely packed structure than the alpha form. The beta form has all of the chain axes oriented in one way as shown in Figure 2, and is triclinic. Both beta-prime and beta forms have an approximate cross-sectional area of about 0.185 nm². These three cross-sectional structures give rise to short spacings as determined by X-ray diffraction and indicated in Figure 3.

The development of the knowledge about polymorphism has not been without controversy. This mainly involved the idea of the existence of a glassy state as proposed by Malkin (1954), but this is now generally accepted as erroneous. A form intermediate between beta prime and beta has been proposed by Hoerr (1960). A more unified and rigorous treatment of polymorphism has emerged in the recent literature (Larsson, 1964, 1966, 1972). Examples of the application of this knowledge to problems in fat crystallization are studies on the phase behavior of hydrogenated fats by Kliner (1971) and studies on cocoa butter and confectionery fats using programmed temperature X-ray diffraction and differential scanning calorimetry by Chapman et al. (1971).

X-ray diffraction patterns can be recorded with a Debye-Scherrer camera or

Fig. 3. Cross-sectional structures of long chain polymorphic modifications. (Source: E.S. Lutton, 1950. Reproduced with permission). d spacings expressed in Angstrom units (Å).

with a diffractometer, the equipment commonly used by crystallographers. The Debye-Scherrer patterns appear as concentric circles on the X-ray film. This type of equipment has the drawback of being usable only at room temperature which does not permit the study of polymorphic transitions as a function of temperature. Special cameras for this purpose have been developed such as the DFR camera used by Aleby (1969) and a particularly useful instrument is the Guinier-Simon camera with integral temperature programming capability (Figure 4). The different polymorphic forms have different melting points and different crystal habits. Another useful X-ray camera is the triple focussing Guinier camera.

Polymorphism and the structure of triglyceride crystals can also be studied by using the electron microscope for electron diffraction of single crystals. This procedure has been demonstrated by Buchheim (1970a) for the study of trilaurin crystals. Methods were developed for preparing suitable crystals of trilaurin, but this procedure may not be suitable for crystals of natural fats. Using this procedure, Buchheim (1970a) found evidence for two beta-prime modifications of trilaurin, one a vertical and the other a tilted form. Two beta modifications of trilaurin were described.

Fat crystals can be observed with the aid of polarized light microscopy. According to Hoerr (1960), crystals of the alpha form appear as platelets of about 5 μm size. Since the alpha form is unstable, it is hard to obtain photographs of these crystals. The beta prime crystals are described as small needles not exceeding one μm in length. Beta
crystals are large, ranging from 20 to 100 um in size and often growing in clumps. Such clumps of large crystals can lead to visually noticeable graininess in fats. The packing of triglyceride molecules in the crystal lattice is influenced by the variety of molecular sizes present in natural fats. The greater the non-uniformity in size, the more difficult it is for the beta form to occur, and the beta-prime form will then be the predominant one. There are natural fats which have relatively uniform fatty acid compositions and these will tend to recrystallize in the beta form. Canola and sunflower oil are examples of this behaviour. Figure 5 shows polarized light micrographs of hydrogenated Canola oil. When this fat was rapidly cooled, the crystal structure included aggregates of up to 10 um (Figure 5A). After tempering for a day at 25 C, there were more small crystals in evidence of up to about 2 um in size (Figure 5B). Ascribing definite polymorphic modifications to fat crystals of specific morphology is probably not justified for many natural fats. This might be applicable in special cases only, such as for highly purified simple triglycerides. The recrystallization of fats into the beta form is a disastrous occurrence when taken place in a consumer product. The product becomes visibly grainy with a mottled color appearance and acquires a crumbly texture (Figure 6) and the crystal structure shows a mass of large needle shaped crystals (Figure 7). This is a good example of how the arrangement of molecules in the crystal lattice directly affects appearance factors such as smoothness and texture. Some selected surface active agents have been found helpful in preventing or delaying the beta-prime to beta transformation in fats. This phenomenon is not well understood and studies are under way to elucidate the mechanism of this action. Recently, Garti et al. (1981, 1982) described the effect of food emulsifiers on crystal structure and habit of
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stearic acid. The crystal habit was examined by using scanning electron microscopy. It was assumed that the stearic acid could serve as a model for the more complex triglyceride mixtures but this is by no means certain. Hernqvist et al. (1981) have described the strong stabilizing effect of 1,2-di-glycerides on the α-triglyceride crystal form.

The polymorphism of fats can also be studied by infra-red spectroscopy, a technique extensively investigated by Chapman (1965) and by deRuig (1977). The major area of interest in the infra-red region is between 690 and 770 cm⁻¹. An example from Woodrow and deMan (1968) (Figure 8) shows the formation of the alpha form on cooling milkfat from 40 to 0 °C and the formation of the beta prime form after holding at 5 °C for ten hours.

Short spacings observed by X-ray diffraction methods are in the order of 0.1 nm. Polarized light microscopy makes it possible to view crystals in the range of 0.5 - 100 μm. For details in the area below 0.1 μm electron microscopy is required. Since fine structures of fats are delicate and temperature sensitive, special techniques are required. Initial electron microscopy studies involved ultra-thin preparations and carbon replica techniques. An example is an electron micrograph of trilaurin crystals produced by Buchheim (1970a) (Figure 9). This figure presents a clear view of the layered architecture of the trilaurin crystal. Much of the work concerned with electron microscopy of fats has been carried out at the German Federal Dairy Research Institute at Kiel and has been mainly focussed on milkfat structure in butter. The work involving fixation, thin sectioning and carbon replica formation produced micrographs showing little more than the outlines of fat globules (Figure 10) (Knoop and Schulz, 1960). More recently the work of this group has involved freeze fracturing, followed by platinum-carbon replica preparation (Buchheim and Precht, 1979; Precht and Buchheim, 1979; 1980; Precht and Peters, 1981). Based on this work, the fat globules in cooled cream have been divided into 4 types based on how much of the fat is in the solid form. Figure 11 represents a fat globule which is stated...
Fig. 10. Electron micrograph of the fat globule structure in butter. (Source: Knoop and Schulz, 1960. Reproduced with permission). S.M. = 1 µm.

to be mostly liquid. However, the highly magnified section appears as a definitely layered structure. Figure 12 is a micrograph of a fat globule with more irregularly formed crystal aggregates. The conclusion of this work is that the amount of solid fat in different fat globules in cream varies widely. This would mean that the fatty acid composition of the fat in the globules would be different, and this is not supported by any previous evidence. Walstra (1976) has discussed the effect of the state of dispersion of fat on its crystallization behavior and on the physical properties of the products. He indicates that emulsified fat needs a much higher degree of supercooling to initiate nucleation, and, therefore, the amount of solid fat in emulsion droplets may be considerably less than in the same fat in bulk form. The microstructure of fat in butter is described by Precht and Buchheim (1980). Two figures from this paper show one area of liquid and crystalline fat (Figure 13) and crystal lamellae of butter after 10 days' storage (Figure 14). The layered structure of this crystalline fat is clearly visible at different magnifications. These authors have attempted to relate the results of the electron microscopy studies to the known rheological behavior of butter. This is a difficult task, especially since the fine structures observed by electron microscopy bear little apparent relation to observations made by polarized light microscopy. The question arises as to whether the rheological properties of fats are more
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Fig. 12. Electron micrograph of a fat globule in cream with solid milkfat. b represents an enlarged section (dotted rectangle) of a. (Source: Buchheim and Precht, 1979. Reproduced with permission). S.M. a = 1 μm, b = 0.2 μm.

Fig. 13. Electron micrograph of liquid (A) and solid (B) fat in 10 day old butter. (Source: Precht and Buchheim, 1980. Reproduced with permission). S.M. = 0.2 μm.

influenced by the internal structure of the crystals than by their ultimate size and pattern of interaction. It is generally agreed that the solid fat content of plastic fats is the major factor determining their rheological behavior. Solid fat content can be measured quantitatively by dilatometry or nuclear magnetic resonance (both wide line and pulsed NMR). It is possible to obtain an idea of the shape and size distribution of crystals by polarized light and electron microscopy. However, these latter techniques are not quantitative. Recently, a method has been developed for determination of the specific surface area of the crystals in a plastic fat by permeametry (deJager et al., 1963, Riiner, 1971). The method is based on the subsidence of the liquid phase of a disperse system under the influence of compression. The compressive force is delivered by a constant vacuum applied to a fat sample. This method promises to be a useful tool in the study of fat microstructure and will hopefully complement the results obtained by microscopic methods. Although the equipment itself is relatively simple, the associated vacuum control instrumentation is not (Figure 15). Riiner (1971) has applied this method to relate polymorphic transitions to crystal sizes in hydrogenated fats. Transition of the beta-prime to beta-modification in hydrogenated sunflower oil reduced the number of crystals by a factor of fifty as determined by the permeametric method.

The objective of studies of the microstructure of fats is to obtain a better understanding of the physical properties, especially rheology, with the aim of better control of such properties.
Fig. 14. Crystal structures of fat in 10 day old butter. (Source: Frecht and Buchheim, 1980). Reproduced with permission. \( S.M.a = 1 \, \mu m, \, b = 0.1 \, \mu m, \, c = 0.1 \, \mu m. \)

Excellent methods for this purpose are now available which should lead to a rapid advancement in our knowledge. However, the usefulness of electron microscopy in this field at this time is limited. It is to be hoped that improved preparation and handling techniques can expand the application of electron microscopy of fats in the future.

Microstructure of emulsions

Emulsions are disperse systems of two immiscible liquids stabilized by an emulsifier or combination of emulsifiers and stabilizers. The importance of associative structures of emulsifiers has been given increasing emphasis and liquid crystalline structures are an important aspect of many emulsions. The International Union of Pure and Applied Chemistry defines an emulsion as follows: "In an emulsion liquid droplets and/or liquid crystals are dispersed in a liquid" (Frisberg, 1976). Food emulsions contain natural emulsifiers and/or synthetic ones. Some food emulsions, especially those based on dairy products, have a fat phase which is not liquid but partly solid and this results in special types of microstructure and properties. Proteins are often the main emulsifiers in food emulsions in many cases in combination with phospholipids and other substances. The original milkfat globule membrane in milk as it leaves the udder has been extensively studied and consists of a complex of several proteins and phospholipids as well as several minor components including enzymes and metals. It is possible to emulsify oils or fats in milk serum (skimmilk) and a stable emulsifier layer is formed almost instantaneously. This also happens when milk is homogenized. The newly formed globule membranes in homogenized milk consist of protein. The conformation of proteins at interfaces and the role of these proteins in stabilizing emulsions has been described by Graham and Phillips (1974). They have visualized the structure of two proteins at the interface (Figure 16). One of
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Fig. 16. Schematic representation of the structures of absorbed films of \(\beta\)-casein and lysozyme at interfaces at different surface concentration. \(\beta\)-casein (disordered, flexible) left column, lysozyme (globular, rigid) right column. Surface concentration top row \(< r_{sat}\), middle row \(\sim r_{sat}\), bottom row \(> r_{sat}\). (Source: Graham and Phillips, 1974. Reproduced with permission).

these is \(\beta\)-casein which has a disordered structure and the other is a globular protein, lysozyme. Protein membranes formed in emulsions containing milk proteins do not have the liquid crystalline structure demonstrated with certain emulsifiers. As a consequence, the globules show no birefringence as is indicated in the polarized light photomicrograph of an emulsion of cottonseed oil in skim milk (Figure 17). The birefringence seen in cream when examined under polarized light at 5 C results from the layers of crystallized milkfat which are formed when the milkfat inside the globules crystallizes (Figure 18). The churning of cream to produce butter involves the destabilization of a limited number of these globules and the expelled liquid fat acts as a cementing material to bind the remaining globules together. For this reason, the microstructure of butter as seen with polarized light is remarkably similar to that of cream (Figure 19). The nature of the birefringent layer of fat globules in milk or cream was clarified by Buchheim (1970b). He used electron microscopy to demonstrate the presence of crystallized fat in the peripheral layers of the globules.

It is now recognized that many emulsifiers interact in a number of ways with both the aqueous and lipid phases and these interactions take the form of liquid crystalline structures or mesomorphic phases. The nature of these liquid crystalline structures has been found to influence viscosity and elasticity of interfacial films with a resultant effect on emulsion stability (Krog.

Fig. 18. Whipping cream as seen in the polarizing microscope at 5 C. S.M. = 5 \(\mu\)m.

Fig. 19. Crystal structure of butter as seen in the polarizing microscope at 5 C. S.M. = 5 \(\mu\)m.
The transformation of a crystallized emulsifier such as a monoglyceride into a liquid crystalline structure has been represented schematically by Krog and Lauridsen (1976) as shown in Figure 20. The major mesomorphic structures are lamellar, hexagonal and cubic. The lamellar structure (Figure 20b) is formed when the emulsifier is heated in the presence of water and alternating bimolecular layers of lipid are separated by layers of water. This structure when examined in the polarizing microscope appears as threadlike striated networks (Figure 21a) and is also known as the neat phase. In addition to polarized light microscopy, the mesomorphic phases can be characterized by their long spacings as determined by X-ray diffraction. The hexagonal phase consists of cylind-
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Fig. 23. Electron micrograph obtained by freeze etching of emulsion, demonstrating the layer structure of a liquid crystal between flocculated droplets. (Source: Friberg et al., 1976. Reproduced with permission).

variety of emulsifiers including monoglycerides, emulsifiers of the Span and Tween type and as shown by Hemker (1981) by tri- and octaglycerol esters. The latter discussed the relationship between the liquid crystalline structures and the functional properties of the emulsifiers in food emulsions.

Food processing often involves formation, destruction, or modification of emulsions, and various forms of microscopy may be used to elucidate the microstructure of the products. An example of the useful combination of scanning and transmission electron microscopy is the study of process cheese microstructure by Rayan et al. (1980). In this case, the protein matrix was visualized by SEM and the nature of the fat globules by TEM. In this way, valuable information about microstructural changes was obtained (Figure 24).

The examples presented in this paper are intended to demonstrate the usefulness of light and electron microscopy in the study of fat and emulsion microstructures, especially when used in conjunction with other techniques such as X-ray diffraction.

Acknowledgement

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References


Fig. 24. Development of microstructure in process cheese in the presence of sodium aluminum phosphate (SALP). 

(a) SEM at 0 time. Large fat particles (F) started to be degraded into smaller particles. Fragment of a calcium phosphate crystal. 

(b) SEM after 10 min in the cooker. Fat is still in the form of large particles, many of which are undergoing emulsification. 

(c) SEM after 40 min in the cooker. Some fat particles (F) are still undergoing emulsification. 

(d) TEM at 0 time. Dark areas are the cheese protein matrix. Light areas indicate fat. 

(e) TEM after 40 min in the cooker. The emulsification process has not been completed and fat particles (F) are still undergoing emulsification. 

(f) TEM detail of one of the added SALP crystals found in abundance during the initial 10 min in the cooker. 

(Source: Rayan et al., 1980. Reproduced with permission).
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Riiner U, Phase behaviour of hydrogenated fats. III. Phase equilibria and crystal sizes, Lebensm. Wiss. Technol. 4, 1971, 139-144.


Discussion with Reviewers

W. Buchheim: The electron micrographs of cream after different thermal treatments clearly show the great variation of fat crystallization and morphology in individual fat globules. This must not necessarily mean that the composition of the butterfat in the globules has to be different, although such evidence has been described by Walstra and Borggreve (Netherlands Milk Dairy J. 20, 1966). The other reason could be that this great variation reflects differences in the degree of supercooling of individual globules due to the absence of crystal nuclei. Please comment.

Author: If in fact this phenomenon will be proven to exist (and not be an artifact of the preparation technique), I would find the latter explanation more plausible than the former.
H. Buchheim: It has been clearly demonstrated that the rheological behaviour of butter made from a given cream is dependent on how crystallized and liquid fat is spatially distributed within the final product. Please comment.

Author: I fully agree with this. However, we still have to explain how the amount of solid fat within a fat globule affects the texture. In butter, we have also fat crystals in the continuous liquid phase. Both types of solid particles are important in determining rheological properties.

D.N. Holcomb: Are the liquid crystal structures shown in Figure 22 consistent with rheological properties of these phases? In general, how important are liquid crystals in determining rheological properties of fat containing foods?

Author: The liquid crystal structures in fat in bulk are definitely consistent with rheological properties. These structures are most important in emulsions and much of the research in this area has been related to emulsion stability.

D.N. Holcomb: Will the author give his opinions as to the future course of studies of the microstructure of fats and emulsions? Can we expect advances in our understanding of these systems through techniques such as low angle X-ray scattering, Fourier transform IR, etc.? Are there any staining techniques that might prove useful in lipid microscopy?

Author: I have tried to emphasize in my paper that this is an extremely difficult area of study. No single technique is adequate and various complimentary techniques should be used. The problem with much of microscopy is that it is not quantitative. More quantitative information is desirable. Low angle X-ray techniques are required for long spacings. Unfortunately, cameras such as the Guinier-Simon are not suitable for this. There is a possibility that suitable modifications to this equipment can be made. Differential staining, e.g. of solid and liquid fat, may be useful in certain cases.

N. Krog: Can you explain in more detail what the Guinier-Simon camera can do? Indicate advantages and limitations in using this camera against others.

Author: The Guinier-Simon camera can provide sharp diffraction patterns of fat samples being temperature programmed in as many as 8 steps over the range of -100 to 100°C. It is not suitable for low angle measurements.

N. Krog: Have you studied the influence of crystal modifiers like sorbitan tri-stearate on the recrystallization problems in Canola margarine?

Author: Yes, we are just concluding the first phase of our study. Sorbitan tri-stearate lowers the melting point, does not affect solid fat content and is effective in delaying the S1 to S2 transition. It also seems to be present in higher concentration in the solid than in the liquid phase of the fat.

N. Krog: It is of interest to know if the fat globules represented in Figs. 11 and 12 have been exposed to different temperature treatment? Can the temperature vary during sample preparation and give cause to the difference between Figs. 11 and 12 rather than the proposed difference in fatty acid compositions?

Author: The possibility of artifacts is an ever present concern. It is more likely, however, that the state of dispersion of the fat influences the rate of crystallization.

N. Krog: Referring to Fig. 19, it would be of interest to know where the milk proteins are located in butter? Are all the proteins in the water droplets or are some of the proteins present on the surface of the fat globules in butter?

Author: Yes, butter contains a proportion of the original fat globules of the cream in an undisturbed condition. The protein membrane of these globules still contain a protein layer. Additional protein is present in the aqueous phase.

N. Krog: Have you done any work with phase contrast or fluorescence microscopy? Or can you give any reference to such studies in relation to food texture?

Author: No, we have not used phase contrast or fluorescence microscopy for work with fats. There appears to be no particular advantage for these techniques in the area of fats, and I know of no recent published work.

F.R. Paulicka: Please comment on the influence of mechanical working (shear, compression) on fat crystal morphology.

Author: The effect of mechanical working on fat rheology is well documented. However, to my knowledge, no body has been able to demonstrate what happens by using microscopy.
MORPHOLOGICAL AND TEXTURAL COMPARISONS OF SOYBEAN MOZZARELLA CHEESE ANALOGS PREPARED WITH DIFFERENT HYDROCOLLOIDS

C. S. YANG AND M. V. TARANTO*

Abstract

The morphology and texture of mozzarella cheese analogs prepared from soy protein isolate, gelatin, fat and different hydrocolloids (gums) were evaluated and compared. The fracturability, hardness and adhesiveness of the cheese analog gels were found to be proportionally related to the amount of fat and gelatin, and concentration and viscosity of gums. However, the stretchability of the cheese analog progels was not controlled by the viscosity of gums, but by the amount of gum and gelatin in the formulation. Fat content affected the fracturability and hardness, but did not have a significant effect on the other textural parameters or stretchability. This physical relationship enabled the preparation of cheese analogs with a broad range of fat contents. Microstructural studies indicated that gums with a lower viscosity formed a uniform and delicate gel network. Gums with a higher viscosity tended to form clumps in the gel network which might retard the alignment of molecules in the progel state and hence, adversely affect the stretching properties of the analog.

Introduction

The development of imitation mozzarella cheese products has progressed rapidly. Most of these products are made from caseinate, a milk protein derivative, which currently is imported and hence the price is expected to remain high. Therefore, it would be advantageous to use several novel, less expensive proteins in the formulation -- such as soybean or peanut proteins -- to replace the caseinate (Taranto and Yang, 1981; Ramamurti et al., 1964; Hannigan, 1979).

Previous reports (Taranto and Yang, 1981; Yang and Taranto, 1982) detailed the development of a mozzarella cheese analog prepared from soy protein, gelatin, fat and gum arabic. These analogs exhibited a typical cheese texture at room temperature. When heated, the analogs melted and stretched in a manner similar to that of natural low moisture-part skim mozzarella cheese.

When used at a concentration of 40% (w/v; 40 g. gum dispersed in 100 ml water), gum arabic induced a pseudoplastic flow behavior in the progel. The analog progel stretchability was similar to that found in melted mozzarella cheese (Taranto and Yang, 1981; Yang and Taranto, 1982). The high solids content of the analog -- composed of gum arabic, gelatin and soy isolate -- was found to induce a "bundle" pattern in the stretched progel similar to natural mozzarella.

However, gum arabic used at a concentration of 40% (w/v) is not only costly but induces a tackiness on the surface of the cheese analog gel. Therefore, the use of other gums and hydrocolloids for the manufacture of cheese analogs was investigated. The results of this investigation are presented here.

Materials and Methods

Raw Materials

Soy protein isolate, Promine-D, was purchased at the start of this research from Central Soya Co. (Fort Wayne, IN). However, since that time, Central Soya has sold the soy isolate business to Archer Daniels Midland (Decatur, IL). A product, Ardex D, similar to Promine D is now being manufactured and sold by Archer Daniels Midland Co. Type B gelatin (128 bloom) was obtained from Baker Chemical Co.
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(Phillipsburg, N.J.). A food grade mixture of powdered xanthan gum-locust bean gum-guar gum (XLG) was obtained from Kelco Co. (Rahway, N.J.) and a powdered guar gum (Jaguar A-40-F) was obtained from Celanese Plastics & Specialities Co. (Louisville, KY). Partially hydrogenated shortening made from coconut oil (Hydro-100) was obtained from Durkee Industrial Foods Group (Chicago, IL).

Sample Preparation and Rheological Evaluations

All samples were prepared and evaluated according to Taranto and Yang (1981) and Yang and Taranto (1982). Details of the sample preparation are given in the two referenced papers.

Textural properties such as fracturability, hardness, adhesiveness, cohesiveness, springiness, gumminess and chewiness of the gel were evaluated using the Instron Universal Testing Machine. Stretchability of the progel is measured with the Weissenberg test (Taranto and Yang, 1981). The progel was the molten or melted form of the cheese analog.

The melting quality test described by Kosikowski (1978) was used in conjunction with other rheological evaluations to investigate the differences among samples prepared with different gums. Melting quality is defined as the amount of radial expansion of a sample disc of known weight and dimensions after heating in an oven for a specified time and temperature. These rheological properties were correlated with microstructural observations.

Sample Preparation for Scanning Electron Microscopy (SEM)

All samples were prepared for SEM according to Taranto and Yang (1981). Samples were fixed, frozen in a liquid nitrogen slush, freeze dried, fat-extracted with chloroform and dry fractured. Samples were then mounted on SEM stubs, coated with gold and examined with a JEOL-JSM-U3 scanning electron microscope operated at 10 kV with a 200 µm aperture and a 13 mm working distance at a 40° tilt.

Statistical Analysis

The F-test procedure (one way analysis of variance) of Steel and Torrie (1960) with a 5% level of significance was used to analyze all data. Multiple comparison of means was performed using Tukey's Q statistic (Steel and Torrie, 1960).

Effect of Gums

Table 1 shows the viscosities of those gums used in this study. Gums such as guar, locust bean, xanthan and XLG form extremely viscous solutions at low concentrations as compared to gum arabic. It is easy to prepare solutions containing up to 40% (w/v) of gum arabic at 25°C and induce a significant thickening effect. The high solids solution is responsible for the excellent stabilizing and emulsifying properties of gum arabic when it is incorporated with a large amount of water-insoluble materials. The use of gum arabic at concentrations up to 40% (w/v) was required to develop a stretchable cheese analog (Yang and Taranto, 1982). However, since soy protein is also an excellent emulsifier, an investigation was initiated into the feasibility of using higher amounts of soy protein as a solids enhancer and a gum system with viscosity similar to gum arabic but at a much lower concentration (such as 0.5-1%).

XLG was chosen primarily because of the synergistic increase in viscosity that results from the mixture of gums and also because of its ability to form a thermoreversible and highly cohesive gel as the colloid concentration is increased. Guar gum was also studied because of its extremely

<table>
<thead>
<tr>
<th>% (W/V)</th>
<th>Gum Arabic¹</th>
<th>Guar Gum¹</th>
<th>Locust Bean Gum¹</th>
<th>Xanthan Gum²</th>
<th>XLG³</th>
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<tbody>
<tr>
<td>0.5</td>
<td>1,389</td>
<td>20</td>
<td>300</td>
<td>380</td>
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<tr>
<td>1.0</td>
<td>-</td>
<td>3,025</td>
<td>59</td>
<td>1,000</td>
<td>1,400</td>
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<tr>
<td>5.0</td>
<td>7</td>
<td>510,000</td>
<td>121,000</td>
<td>-</td>
<td>(gel)</td>
</tr>
<tr>
<td>30</td>
<td>200</td>
<td>-</td>
<td>-</td>
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<td>50</td>
<td>4,163</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

¹Glickman, 1962
²Rocks, 1971
³Kelco Company
SOYBEAN CHEESE AND GUMS

Table 2: Formulations for soybean mozzarella cheese analogs.¹

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Gum, (g) (%)²</th>
<th>Gelatin (g) (%)</th>
<th>Soy Protein (g) (%)</th>
<th>Fat (g) (%)</th>
<th>Water, (g.) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5 XLG (0.28)</td>
<td>20 (11.1)</td>
<td>40 (22.2)</td>
<td>20 (11.1)</td>
<td>100 (55.4)</td>
</tr>
<tr>
<td>2</td>
<td>1 XLG (0.55)</td>
<td>30 (16.6)</td>
<td>30 (16.6)</td>
<td>40 (19.9)</td>
<td>100 (49.8)</td>
</tr>
<tr>
<td>3</td>
<td>1 XLG (0.55)</td>
<td>30 (16.6)</td>
<td>30 (16.6)</td>
<td>40 (19.9)</td>
<td>100 (49.8)</td>
</tr>
<tr>
<td>4</td>
<td>1 XLG (0.50)</td>
<td>30 (14.9)</td>
<td>30 (14.9)</td>
<td>40 (19.9)</td>
<td>100 (49.8)</td>
</tr>
<tr>
<td>5</td>
<td>1 guar (0.55)</td>
<td>30 (16.6)</td>
<td>30 (16.6)</td>
<td>40 (19.9)</td>
<td>100 (49.8)</td>
</tr>
<tr>
<td>6</td>
<td>40 gum arabic (19.0)</td>
<td>40 (19.0)</td>
<td>20 (10)</td>
<td>10 (4.3)</td>
<td>100 (43.5)</td>
</tr>
<tr>
<td>7</td>
<td>40 gum arabic (17.4)</td>
<td>40 (17.4)</td>
<td>20 (10)</td>
<td>10 (4.3)</td>
<td>100 (43.5)</td>
</tr>
</tbody>
</table>

¹Samples were prepared according to the procedure described by Yang and Taranto (1982).
²As-is percentage based on total formula weight.

high viscosity at low concentrations (Table 1).

Effect of Gelatin and Soy Protein

Both gelatin and soy protein form thermoreversible gels at a certain concentration and temperature range. Although they are not stretchable in the progel state, they may have a synergistic effect on the progel stretchability in the presence of gums. Hence, different concentrations of these two proteins were studied (Table 2).

Effect of Fat

Fat was found to enhance the hardness of the cheese analog gel as well as the mouthfeel and heat meltability (Yang and Taranto, 1982). Therefore, we investigated the effect of fat incorporation on the texture, stretchability and melting quality using mixtures in which the solids content was reduced by replacing the 40% (w/v) gum arabic with 1% (w/v) of other gums (Table 2).

Results and Discussion

Composition data on samples made from different gums in addition to a commercial mozzarella cheese are listed in Table 2. Results of the Instron texture profile analysis (TPA), Weissenberg test, melting quality of these samples and several effects of the key ingredients are listed in Tables 3-6. Morphological comparisons are illustrated in Figures 1-8.

Effect of Gums

Samples prepared with 0.5 g. XLG (sample 1 in Table 2) and 1 g. XLG (sample 2 in Table 2) were compared with samples prepared with 40 g. gum arabic (samples 6 and 7 in Table 2). Although a 1% (w/v) XLG solution had a similar viscosity to a 40% (w/v) gum arabic solution (Table 1), cheese analog samples prepared with 40 g. gum arabic had

Figure 1 - Scanning electron micrograph of mozzarella cheese analog prepared from 40% gum arabic, 10% fat, 40% gelatin, and 20% soy isolate (Sample 6 in Table 2). Note the soy protein and gum particles (SP). AC-air cell.
Table 3: Objective evaluations of samples made with XLG or gum arabic.1

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1Each value is a mean ± S.D. (n=4). Means in the same column with different letters are significantly different (p=0.05).

2Samples described in Table 2; samples 6 and 7 were external controls.

Figure 2 - Scanning electron micrograph of mozzarella cheese analog prepared from 0.5% XLG, 20% fat, 20% gelatin, and 40% soy isolate (sample 1 in Table 2). Note the protein "chunks" (indicated by the arrows). AC-air cell.

Figure 3 - Scanning electron micrograph of mozzarella cheese analog prepared from 1% XLG, 20% fat, 20% gelatin, and 40% soy isolate (sample 2 in Table 2). Note that there are no "chunks". AC-air cell.

significantly higher values for the fracturability, hardness, gumminess and chewiness (Table 3). This could be attributed to the higher solids content of gum and gelatin in samples 6 and 7 compared to that in samples 1 and 2 (Table 2).

The amounts of fat and soy protein isolate in the gum arabic samples were lower than those in the XLG sample (Table 2). Therefore, one would have expected higher TPA values for the XLG samples. Samples prepared with 0.5 g. and 1 g. XLG had similar TPA values (Table 3). SEM micrographs revealed that samples prepared with gum arabic exhibited some large gum particles with adhering soy protein (Figure 1). This was due to the slight salting-out effect with gelatin and gum arabic. These exuded gum arabic-soy protein particles are believed to be the cause of the tackiness on the surface of the cheese analog. Samples prepared with XLG (Figures 2 and 3) showed no visible gum particles. The gel surfaces were not tacky. The 0.5 g. XLG product (sample 1 in Table 2) exhibited numerous soy protein "chunks" in the gel matrix (Figure 2). No structure of this nature was observed in the 1 g. XLG product (sample 2 in Table 2; Figure 3).

It is theorized that the protein chunks in the 0.5 g. XLG product resulted from an...
SOYBEAN CHEESE AND GUMS

Table 4:
Objective evaluations of samples made with different gums.

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Each value is a mean ± S.D. (n=4). Means in the same column with different letters are significantly different (p=0.05).

Samples described in Table 2; samples 6, 7 and C were external controls.

Figure 4 - Scanning electron micrograph of mozzarella cheese analog prepared from 1% XLG, 20% fat, 30% gelatin, and 30% soy isolate (sample 3 in Table 2). This micrograph was prepared from the sample in the stretched state, note the clear, fiber-type alignment in the stretched bundle.

Figure 5 - Scanning electron micrograph of natural mozzarella cheese (low moisture part-skim). This micrograph was prepared from sample in the stretched state. Note the clear, fiber-type alignment in the stretched bundle.

An insufficient amount of XLG to assist the soy protein in forming a honeycombed gel network with gelatin. The absence of chunks in the 1 g. XLG product (Figure 3) supports this explanation.

The condensed areas in the protein matrix of the 0.5 g. XLG product formed a slightly (though not statistically significant, Table 3) harder gel compared to the 1 g. XLG product. These condensed areas also prevented the alignment of the molecules during stretching and therefore, the Weissenberg test indicated a zero stretchability for the 0.5 g. XLG product (Table 3).

The morphology of the 1 g. XLG analog (sample 3 in Table 2) in the melted and stretched state is shown in Figure 4. The fibrous elements are formed into a large bundle approximately 20 µm in width. The structural features of the melted and stretched natural low moisture-part skim.
Table 5:
Objective evaluations of samples made with different levels of gelatin and soy isolate.1

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1Each value is a mean ± S.D. (n=4). Means in the same column with different letters are significantly different (p=0.05).
2Samples described in Table 2; sample C was an external control.

Figure 6 - Scanning electron micrograph of mozzarella cheese analog prepared from 1% guar gum, 20% fat, 30% gelatin, and 30% soy isolate (Sample 5 in Table 2). Note that a non-uniform honeycombed protein network is formed. AC-air cell.

Figure 7 - Scanning electron micrograph of mozzarella cheese analog prepared from 1% XLG, 20% fat, 30% gelatin, and 30% soy isolate (Sample 3 in Table 2). Note the uniform, honeycombed protein network. AC-air cell.

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mozzarella cheese are shown in Figure 5. There is an alignment of the fibrous elements with the fibers ranging from 0.5-6 \( \mu m \) in width. The stretched protein matrix shows numerous small voids which are separated by thin membranes (Figure 5). There was no significant difference in the stretchability between the 1 g. XLG analog (sample 3 in Table 2) and the natural mozzarella cheese (Table 4). Therefore, it appears that the formation and alignment of fibrous elements in the melted and stretched product is required for the analog to exhibit a stretchability equal to that of natural mozzarella cheese.

A 1% (w/v) guar gum solution has a viscosity about triple and double that of 40% (w/v) gum arabic and 1% (w/v) XLG solutions, respectively (Table 1). Analogs prepared with 1 g. guar gum (sample 5 in Table 2) had significantly higher values for fracturability and hardness than the analogs prepared with 1 g. XLG (sample 3 in Table 2) or commercial mozzarella cheese (Table 4). Adhesiveness of sample 5 (guar) was the highest among the samples prepared with different gums and was comparable to that of the commercial mozzarella sample (Table 4). Cohesiveness showed no significant difference except for the lower value of the 1 g. XLG analog (sample 3) (Table 4). There was no detectable stretchability in...
SOYBEAN CHEESE AND GUMS

Table 6: Objective evaluations of samples made with different fat contents.

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1 Each value is a mean ± S.D. (n=4). Means in the same column with different letters are significantly different (p=0.05).
2 Samples described in Table 2; sample C was an external control.

Figure 8 - Scanning electron micrograph of mozzarella cheese analog prepared from 1% XLG, 40% fat, 30% gelatin, and 30% soy isolate (Sample 4 in Table 2). Note that the size of the network voids is much smaller and the protein matrix is denser than in sample 3 (Figure 7). AC-air cell.

The highly viscous guar gum analog (sample 5, Table 4). The very dense protein matrix of the 1 g. guar analog (sample 5, Figure 6) is similar to the matrix seen in the 0.5 g. XLG analog (sample 1, Figure 2). Neither of these two samples had a stretchability that could be measured with the Weissenberg test.

The condensed protein matrix, in some manner, must prevent the formation and alignment of the fibrous elements when the analogs are melted and stretched. Without the formation of the fibrous bundles, the product will not exhibit the stretching and stringing characteristics of melted mozzarella cheese.

Effect of Gelatin and Soy Protein

Cheese analog samples 2 and 3 each contained the same amount of XLG and fat solids (Table 2). Sample 3 contained equal portions of gelatin and soy protein, whereas sample 2 contained gelatin and soy protein in a 1:2 ratio (Table 2). The TPA results indicated that sample 3, which had TPA values similar to the commercial mozzarella cheese, had almost twice the fracturability, hardness and stretchability as sample 2 (Table 5). This clearly indicates that gelatin contributed to both the gel texture and progel stretchability. The higher thermoreversible character of a gelatin gel (compared to a soy protein gel) enhanced the melting quality of sample 3 (Table 5).

No distinct structural difference was detected between sample 2 (Figure 3) and sample 3 (Figure 7). The rigidity of the resultant gels appears to be dependent upon the concentration ratio of gelatin and soy protein when all other ingredients are held at a constant level.
Effect of Fat
Increasing the amount of fat while holding the level of other ingredients constant (sample 3 and 4 in Table 2) was found to significantly increase the fracturability and hardness of the XLG cheese analog (Table 6). The stretchability and melting quality of the analog were significantly decreased at higher fat levels (Table 6).

The increase in hardness is due to the reinforcement effect of the additional fat in the gel system (Stainsby, 1977; Yang and Taranto, 1982). The XLG analog prepared with a higher fat level (sample 4 in Table 2) is shown in Figure 8. The additional fat appears to have disrupted the uniformity of the honeycombed protein matrix (Figure 8). The size of the network voids has been reduced compared to the XLG analog with a lower fat content (sample 3 in Table 2; Figure 7). This decrease in size of the voids resulted in a denser protein network (a greater amount of solids per unit volume). This increase in density resulted in a higher gel resistance (rigidity) and lower stretchability. These data are in agreement with Yamano et al. (1981) who concluded that fat delayed soy protein gelation which resulted in a fine and hard gel structure.

Conclusions
Cheese analogs prepared with XLG were found to be the most similar to natural low moisture-part skim mozzarella cheese. In particular, sample 3 (Table 2) had the best match with mozzarella cheese in both the gel and progel states. Most important was that the XLG analogs were not tacky on the gel surface compared to the very tacky surface of the gum arabic analogs. The concentration ratio of gelatin and soy protein was found to significantly affect the gel and progel characteristics. Cheese analogs made with an equal proportion of gelatin and soy protein (sample 3 in Table 2) were found to have the best match with the TPA values and stretchability of mozzarella cheese.

References
Rasamurti K. Sreenivasamurthy V, Johar DS. (1964) Preparation of cheese-like products from peanut and biochemical changes that take place during their ripening. Food Tech. 6:98-100.


DISCUSSION WITH REVIEWERS
D. N. Holcomb: In the introduction you cite the (price) advantage of using "less expensive proteins...such as soybean..." The formulations shown in Table 2 rely heavily on gelatin. Is it a "less expensive" protein?
Authors: The current price for soy protein isolate is about $1.10/pound and gelatin about $2.15/pound. Refinement of our formulation to reduce the amount of gelatin is necessary to reduce the overall ingredient cost. We have studied a few other gelling agents, but none have performed as well as gelatin.

D.N. Holcomb: What are the organoleptic qualities of these products? Do taste panels agree that sample 3 is the most similar to natural mozzarella cheese?
Authors: The analogs as we prepare them have a very bland flavor. At this time, we have not run any formal sensory panels to compare products. We concluded that sample 3 was the most similar to mozzarella cheese based on our morphological and textural data.

K. Sato: Would you please explain what structural features of melted and stretched cheese are associated with "fiber alignment?"
Authors: When our cheese analog and natural mozzarella cheese is melted and stretched, the protein matrix in both products is elongated into large parallel fibrils. In the case of our cheese analog, these fibrils are interlaced in a rope-like fashion. In the natural mozzarella cheese, the fibrils appear to be crosslinked by a network of fine fibrils. In both cases, the fiber alignment we refer to is the parallel array of the large (coarse) fibrils.

W. J. Wolf: How did you ascertain that the large particles in the gum arabic containing analog (Figure 1) were gum arabic and that the adhering material was soy protein?
Although you attribute tackiness on the surface of the analog to the "gel particles" did you actually observe such particles on unfractured surfaces?

Authors: The large particles and adhering material were differentiated on the basis of their size and morphology. The morphology of the adhering material closely parallels the characteristics of soy protein particles reported by A. Hermansson (J. Amer. Oil Chemists Soc. 56: 275, 1979). We did not observe the "gel particles" on unfractured surfaces.

W. J. Wolf: What is the basis for attributing a "salting out" effect to gelatin and gum arabic in regard to the "adhering soy protein" on the large particles?

Authors: We believe this effect is due to a competition for the limited water available during the heat treatment phase of the analog manufacturing procedure. The soy proteins do not appear to effectively compete with the gelatin and gum arabic for the limited hydration water.

M. Kalab: Which polysaccharides in the various gums are responsible for the high viscosities of their solutions?

Authors: The viscosity of the various gum solutions is due to the structural features and molecular weight distribution of their respective polymers. We refer you to the textbook entitled: "Industrial Gums - Polysaccharides and their Derivatives," R. L. Whistler, Ed., Academic Press, 1959, for further details.

M. Kalab: Was the microstructure of gelatin and soy protein mixtures studied in the absence of gums? If yes, how did non-stretchable structures differ from the stretchable ones made with the gums?

Authors: No, we did not study the microstructure of gelatin/soy protein gels in the absence of gums. We did study the textural properties of such systems. A cheese analog can be made from gelatin and soy protein without gums. However, an excess of gelatin is required for the system to exhibit a texture which simulates a natural cheese. The system will melt when heated, but it is not stretchable.

D. A. Froehlich: Even though all formulations for the soybean mozzarella cheese analogs included 100 g. water, the final moisture content of the cheeses would have covered quite a wide range (approximately 43 to 55%). Was any consideration given to the effect of moisture content of cheeses to the TPA values?

Authors: Moisture content is indeed an important factor in TPA evaluations. In these experiments, we tried to keep the moisture content of the analogs prepared with the same ingredients constant (see samples 1, 2, 3 in Table 2). Except for sample 7 (Table 2), all the remaining analogs ranged between 48 to 55%. This range of moisture content was taken into account in the initial design of our experiment. Our initial data indicated a minimal effect on the TPA values over this range of moisture. We have recently completed a more detailed study on the effect of moisture content on the cheese analog TPA values. These data will be discussed in our next paper.
Electron Microscopic Localization of Solvent-Extractable Fat in Agglomerated Spray-Dried Whole Milk Powder Particles

Wolfgang Buchheim

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Abstract

An agglomerated spray-dried whole milk powder has been studied by electron microscopy before and after extracting approximately 10% of total fat with petroleum ether at 25°C for 1 hour. The powder samples were suspended in polyethylene glycol, cryofixed and further prepared by the freeze-fracturing technique. These studies demonstrate that the solvent-extractable fat (the so-called 'free fat') consists partly of surface fat and partly of fat extracted from fat globules within the powder particles. The spatial distribution of such solvent-accessible fat globules appeared to be rather uneven, i.e. whereas certain limited volumes within a powder particle showed an almost complete extraction, others remained unaffected. There were no indications that fat globules near the periphery of the powder particles were more accessible for the solvent than those in the interior of the particles. The results of this study generally confirm the model by Buma for the distribution of 'free fat' in dried milk.

Introduction

Fat-containing powders such as, e.g. cream powder or whole milk powder, generally lose a certain portion of their fat when treated with organic fat solvents. The amount of extractable fat, the so-called 'free fat', is not only dependent upon the extraction conditions (type of solvent, temperature, time, mechanical movement etc.) but is also strongly influenced by manufacturing and storage conditions of the powder. Generally, high 'free fat' values have negative effects on such powder properties as e.g. storage stability, wettability and dispersibility, and stickiness (1-6).

In a very detailed and comprehensive study, Buma (4) investigated aspects of the quantitative determination of 'free fat', relation of the 'free fat' to the structure of the powder particles, to processing and storage conditions, and to other physical properties of the powders. He proposed a detailed model for the distribution of 'free fat' in spray-dried milk. According to this model, 'free fat' consists of fat found on the outer surface of the powder particles and of fat which is accessible for solvent extraction in the interior of the particles. By studying the dependence of 'free fat' on extraction time it has been found that the surface fat is removed almost instantaneously whereas the extraction from the interior is a slow diffusion-like process (1, 4).

Light microscopy can be applied to visualize surface fat on milk powder particles after staining with osmium tetroxide vapour (3).

Electron microscopical evidence for thin layers of fat on the surface of milk powder particles has been first described by Müller (9) using thin-sectioning. By freeze-fracturing powder suspensions in non-aqueous media it should be possible to more precisely localize 'free fat' in dried milk products (2). By applying this technique to a spray-dried whole milk powder before and after extraction with a fat solvent it was attempted to obtain direct evidence for the distribution of the 'free fat'.

Material and Methods

For this study a commercially available agglomerated spray-dried whole milk powder, not coated with a lecithin-fat mixture, was used. Its fat content was approximately 26 g per 100 g of powder.
The exact processing conditions of this powder, however, could not be ascertained.

'Free fat' was extracted according to a procedure described earlier (1). 500 mg of the powder was added to 50 ml petroleum ether (boiling range 40-60°C), allowed to stay in it for 1 h at 25°C, and was gently shaken every 10 min. Thereafter the solvent was separated by paper filtering and the powder particles were allowed to dry in air for 15 min at room temperature.

The preparation for electron microscopy followed the procedure described elsewhere (2). The original and the extracted powders were mixed with polyethylene glycol (molecular weight 400) in amounts to produce highly viscous yet flowing suspensions. Small amounts (1-2 μl) of the suspensions were transferred onto normal freeze-fracture specimen holders (Balzers). The specimens were cryo-fixed by immersion into melting Freon 22 (-160°C). Freeze-fracturing was carried out in a Balzers BA 360 M unit. The specimens were cleaved at -110° C and replicated by shadowing with 2 nm platinum/carbon at 45 degrees and a subsequent evaporation of 15 nm carbon using electron guns. For cleaning the replica film, the specimens were at first carefully immersed in pure polyethylene glycol, the replicas were then stepwisely transferred onto the surface of pure water. The milk powder constituents were removed by a treatment in a sodium hypochlorite solution and in acetone, using pure water as an intermediate medium.

Results and Discussion

The extraction procedure resulted in a loss of 2.45 g ‘free fat’ per 100 g of milk powder or 9.5 g ‘free fat’ per 100 g fat.

When suspensions of spray-dried milk powders in polyethylene glycol are freeze-fractured, either cross-fractures through individual powder particles or fractures along the boundary surface between the particles and polyethylene glycol are produced. However, it could be observed that surface fractures occurred preferentially only if the powder particle had been fractured out of the polyethylene glycol matrix leaving behind its surface relief as a counterpart. From a comparison of numerous micrographs showing surface views of the powder particles, it could not be concluded with certainty whether the cleavage occurred only between the outermost layer of the surface fat and the polyethylene glycol matrix or whether occasionally this superficial fat was also cleaved internally along its individual monolayers.

Such surface views of particles of the original powder frequently revealed the typical morphology of thin layers of crystallized fat (Fig. 1 and 2). The distribution of such surface fat is rather uneven. One can find powder particles which are more or less entirely covered with fat (Fig. 1) whereas others are devoid of fat. These layers of surface fat exhibit a high degree of crystalline order (Fig. 2) which is characteristic for high-melting fractions of milk fat. Occasionally layers of surface fat can also be detected at the periphery of cross-fractured powder particles as shown in Fig. 3. This micrograph demonstrates also the local occurrence of the surface fat as crystalline aggregates consisting of several monolayers, each approximately 5 nm thick.

Whereas the particle surfaces of the solvent-treated milk powder appeared largely devoid of any fat deposits, the effect of this treatment on the internal fat phase, i.e., the finely dispersed fat globules, became very obvious in the cross-fractures of the particles. In the respective micrographs, one can clearly distinguish between fat globules which have not been affected by the solvent and those which have been completely extracted leaving empty cavities.

Generally it was observed that solvent-accessible fat globules are rather unevenly distributed within the powder particles. Fig. 4 shows an apparently small powder particle (diameter of the cross-sectional area visible, 7-8 μm), where the
Fig. 3. View (cross-fracture) of a local accumulation of surface fat (sf) at the interface between the milk powder particle (mp) and polyethylene glycol (peg). cm: casein micelles. Bar, 0.5 μm.

Fig. 4. A smaller milk powder particle after the treatment with the fat solvent. Empty cavities (c) represent extracted fat globules. Fg: still intact fat globules. Bar, 1 μm.

Fig. 5. Peripheral part of a milk powder particle after extraction of 'free fat'. Note the high percentage of intact fat globules in that area. Bar, 1 μm.

Fat of most fat globules has been removed by the solvent. The background structure within these empty globules has a slightly undulating appearance. It seems that at least some of these extracted fat globules are directly adjacent to each other.

In contrast to this high degree of local fat extraction one can easily find powder particles or at least parts of particles where the solvent obviously had no access to the fat globules. Such intact fat globules occurred in more central parts as well as directly near the periphery of the powder particles. Fig. 5 shows such an area near the surface of a particle. Fig. 6 is another example of a more peripheral area of a larger powder particle having lost a considerable amount of fat during extraction. The characteristic feature in that micrograph (even more easily distinguished in Fig. 7) is the fact that polyethylene glycol has partly penetrated into the cavities of the extracted fat globules. The amorphous fine structure of that embedding medium enables the differentiation from the fat phase.

Summarizing the overall observations on the degree of fat extraction from the interior of the powder particles it is striking that it occurred preferentially in limited areas. Local accumulations of 'empty' fat globules often alternated with apparently intact areas in the immediate vicinity.

Finally it should be mentioned that the micrographs of cross-fractured powder particles did not exhibit the fine capillaries which necessarily must exist in order to allow the extraction from the individual fat globules. One possible explanation could be that such capillaries are very narrow and are perhaps filled with polyethylene glycol.

The structures in Fig. 6 and 7 demonstrate clearly that the spaces left by extracted fat globules can take up the embedding medium.

The results described above confirm the model of Buma (4) concerning the origin and distribution of 'free fat' in dried milk. According to this model, the solvent-extractable portion of the total fat results from 4 different sources: (I) 'surface fat'; (II) 'outer layer fat', i.e. fat from fat globules in the surface layer which are directly exposed to the solvent; (III) 'capillary fat', i.e. fat from fat globules in the interior of the powder particles, which is extracted through capillary pores or cracks, and (IV) 'dissolution fat', i.e. fat from internal fat globules which can be reached by solvents via the holes left by dissolved fat globules in the outer particle layer or close to wide capillaries in the powder...
Fig. 6. Peripheral part of a milk powder particle after the extraction of ‘free fat’. Note the high percentage of extracted fat globules (c). Bar, 1 μm.

From the observed distribution of extracted fat globules within powder particles it is suggested that the so-called ‘dissolution fat’ component contributes a high proportion to the ‘free fat’ extractable from the interior of the milk powder particles.

Acknowledgment

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References


Fig. 7. Part of Fig. 6 demonstrating that polyethylene glycol (peg) is able to partly penetrate extracted fat globules. Bar, 1 μm.


Discussion with Reviewers

R.J. Carroll: Have you examined an extracted non-agglomerated whole milk powder? How would the electron microscopic appearance of this product compare with that of the agglomerated milk powder observed in this study?

Author: During a few preliminary examinations of extracted non-agglomerated whole milk powders we made similar observations as with the agglomerated powder. A very careful qualitative and quantitative evaluation of a greater number of micrographs will probably be necessary to completely characterize different whole milk powders. A major problem will be that the freeze-fracture technique allows only single cross-sectional areas of powder particles to be inspected.
localization of fat in milk powder particles

M. Kalab: Was the loss of fat from the milk powder particles due to extraction with petroleum ether gradual or was it in steps corresponding to the main powder, the presence or absence of vacuoles etc.?

Author: From the several studies we made so far, a definite answer cannot be given. I would assume, however, that there is a relationship between the amount of surface fat on individual powder particles and their physical properties (size, internal structure etc.) which has to be revealed by systematic comparative studies.

J. de Vilder: You mentioned that the high degree of crystalline order of the layers of surface fat is characteristic of high-melting fractions of milk fat (Fig. 2). Does this mean that in general the composition of the 'free fat' is different from the original composition of the milk fat?

Author: According to some preliminary measurements of the index of refraction and the fatty acid composition of the surface fat and the total fat it is concluded that there is some fractionation with the accumulation of higher-melting fat fractions at the surface of the powder particles.

J. de Vilder: It is a common idea that, besides the fact that the solvent has to be able to come into direct contact with the fat, one only has 'free fat' if also the fat globules are not completely covered with an intact fat globule membrane. Is it possible to find a confirmation for this theory in the presence or absence of fat globule membranes in this work?

Author: Although the micrographs were inspected carefully, no evidence could be obtained on the degree of integrity concerning the membranes of extracted fat globules. The sites at which the solvent came into direct contact with the fat phase are somewhat obscured similar to fine capillaries and cracks within the powder particles.

J. de Vilder: It is described that local accumulations of 'empty' fat globules often alternated with apparently intact areas in the direct vicinity. Is there any explanation for this observation?

Author: The only explanation I can give is that the solvent probably penetrates into the powder particle along fine capillaries and cracks and only dissolves the fat from those fat globules which are accessible along such channels.

J. de Vilder: In this work, a commercially available agglomerated spray-dried milk powder has been studied. Does the author think that the same result and conclusions would be obtained if different kinds of whole milk powders would be studied? The influence of homogenization of the concentrate would be particularly interesting in this respect.

Author: I would assume that a careful comparative study of whole milk powders manufactured in different ways would result in characteristic differences, especially if the extraction curves of such powders show distinct differences.

D.H. Bullock: There seems to be considerable inconsistency as to which areas of the particles are attacked by the solvent, that is, why are some peripheral areas inaccessible. Is it possible that some cavities which have been interpreted as having had the fat removed by the solvent are cavities resulting from a breaking out of the fat globule at time of fracture owing to the fracture occurring at the globule membrane or interface?

Author: The appearance of freeze-fractured milk powders before and after extraction makes it rather unlikely that some of the cavities in the extracted samples resulted from a breaking out of the fat globules during cleaving.

D.H. Bullock: Could the inconsistency be due in part to the lactose being crystallized in some areas and amorphous in others following the instantizing process? (I have presumed that agglomeration was the result of a rewetting and redrying instantizing process.)

Author: Since crystallization of lactose could not be observed in areas largely extracted by the solvent, it remains questionable whether a local crystallization of lactose is responsible for the inhomogeneous extraction of fat.

T.J. Burna: Is it possible that during the freeze-fracturing, one part of the fat globules remains in one half and another part of the fat globules remains in the other half of the sample? If it is not impossible, at least a part of the cavities (empty fat globules) could be an artifact due to the fracturing process.

Author: Although the possibility exists that a fat globule is fractured at its periphery, the typical appearance of the cavities, i.e. empty fat globules, enables a clear identification. This differentiation is, of course, easier to be made with larger than with very small fat globules.

M. Kalab: You have mentioned that the background structure of empty fat globules in the extracted milk powder particles has a slightly undulated appearance. This is particularly well evident in the globule below the centre in Fig. 4. Do you assume that the 'bumps' are casein micelles? What is the material binding them?

Author: It is somewhat difficult to understand and interpret this peculiar structure of the inner surface of empty fat globules. Since the fat globules of this powder are homogenized fat globules which have casein micelles and submicelles adsorbed at their surface as a secondary membrane, the 'bumps' could indeed represent casein particles. But I would assume that the drying process itself plays a role in the development of this peculiar fine structure.

M. Kalab: It is interesting to observe that some cavities after fat extraction are filled with polyethylene glycol in large dry milk particles whereas no cavities are filled in the small particles shown in Fig. 4. Have you studied the conditions for a complete impregnation of the powder particles with polyethylene glycol?

Author: Although I have not yet studied the conditions for a complete impregnation with polyethylene glycol, I would assume that only those cavities are filled quickly which have wider openings through which polyethylene glycol can flow in and the entrapped air can escape.

M. Kalab: Was the loss of fat from the milk powder particles due to extraction with petroleum ether gradual or was it in steps corresponding to the individual kinds of fat in the product? Could you please show a diagram obtained by plotting the amount of fat extracted versus time to show the rate of extraction?
Author: Buma (4) has demonstrated that the loss of fat with increasing extraction time showed considerable variation between different powders and depended also on the extraction conditions. During extraction, one 'step' occurs only at the very first beginning, i.e. when the surface fat is removed, thereafter the loss of fat is a rather slow process (1). The following diagram demonstrates the observed differences between a non-agglomerated (x) and an agglomerated (●) spray-dried whole milk powder.

Fig. 8. Extraction of fat with petroleum ether from a non-agglomerated (x) and an agglomerated (●) spray-dried whole milk powder. Extraction was carried out at 25°C.

\[ \text{EXTRACTED FAT (G/100G POWDER)} \]

\[ \text{EXTRACTION TIME (min)} \]
LITERATURE ABSTRACTS

The purpose of this new section is to provide readers of FOOD MICROSTRUCTURE with abstracts of papers on food microscopy and related subjects. Abbreviations LM, EM, SEM, and TEM stand for light microscopy, electron microscopy, scanning electron microscopy, and transmission electron microscopy, respectively. The abstracts are either author's own or have been prepared by the editors. FOOD MICROSTRUCTURE invites abstracts from other journals; each abstract should bear the original title, with translation in English where applicable, names of all the authors, address of the senior author, full (unabbreviated) name of the journal, year, volume, issue (if available), and inclusive pages. Please contact the authors directly for reprints of articles. These abstracts have been organized in no particular order. Please contact Dr. Kalab if you wish to submit abstracts for FOOD MICROSTRUCTURE.

MICROSTRUCTURE OF VARIOUS CHEMICAL COMPOUNDS CRYSTALLIZED IN CHEDDAR CHEESE.


Tyrosine, sorbic acid, Ca lactate, Ca phosphate, and NaCl, which have been reported to occur as crystals on various cheeses, were induced to crystallize on the surface of mild Cheddar cheese. The crystals were examined by SEM and photographed at magnifications ranging from 160x to 16,000x.

OBSERVATION OF FAT DISTRIBUTION IN CHEESE BY INCIDENT LIGHT FLUORESCENCE MICROSCOPY.


A combined cheese tier and microslide were constructed from stainless steel and used to prepare cheese samples (Ø 7 mm) for fluorescence microscopy. Freshly exposed cheese surface was stained at 4°C with an aqueous mixture of 2 parts 0.1% Acridine Orange and 1 part 0.1% Phosphine for 20 min and then flushed with a fresh application of the staining solution. Excess stain was removed with a piece of filter paper. Using a BG 12/6 mm exciter filter and an orange screen filter, fat appeared as yellow-green regions on the background of protein coloured red-brown. Curd granule junctions in Cheddar cheese were discernible as interfaces of protein devoid of fat. In Cheddar cheese made from ultrafiltered milk the fat was present in globules considerably larger than those in control cheese.

ULTRASTRUCTURES OF BACTERIOPHAGES ACTIVE AGAINST STREPTOCOCCUS THERMOPHILUS, LACTOBACILLUS BULGARICUS, LACTOBACILLUS LACTIS AND LACTOBACILLUS HELVETICUS.


Several strains of phages active against Streptococcus thermophilus and species of Lactobacillus were examined with an electron microscope after negative staining with phosphotungstic acid or uranyl acetate. S. thermophilus bacteriophage exhibited exceptionally long tails (polytails). The width and structure of the polytail was the same as a normal phage tail, 10 nm, but was 2 to 4 times longer, 480-960 nm. Preparations revealed extensive adsorption of S. thermophilus bacteriophage to broken bacterial cell walls. One strain of S. thermophilus phage had a spherical structure at the posterior end of its tail. The bacteriophages of Lactobacillus bulgaricus and Lactobacillus helveticus had a distinct contractile tail sheath, whereas Lactobacillus lactis phage did not.

DETECTION OF CURD GRANULE AND MILLED CURD JUNCTIONS IN CHEDDAR CHEESE.


Curd granule junctions and milled curd junctions in Cheddar cheese were visualized by slicing the cheese, fixing the slices in a glutaraldehyde solution, dehydrating them in alcohol, defatting in m-hexane, drying, and sanding with a carburendum paper. The sections were photographed with a 35-mm camera equipped with bellows. The junctions consisted of fat-depleted areas which formed the borders between adjoining curd granules. The curd granules were smooth and had regular shapes and uniform dimensions in stirred-curd cheese made by cutting the curd with wire knives in the traditional way. Cutting of the curd with the stirring mechanism instead of knives produced ragged, irregular, and nonuniform granules. A model has been suggested, which explains the formation of the curd granule junctions and their changes during cheddaring. Cheddar cheese was characterized by the presence of another kind of junctions called milled curd junctions.

CURD GRANULE AND MILLED CURD JUNCTION PATTERNS IN CHEDDAR CHEESE MADE BY TRADITIONAL AND MECHANIZED PROCESSES.


Various cheese making practices produced characteristic patterns of curd granule and milled curd junctions in Cheddar cheese. Using such patterns, the amount of flow during cheddaring was compared in cheese made by traditional, semi-mechanized, and four automatic cheddaring methods. Products from mechanized systems (Damrow Draining and Matting Conveyor, Bell-Siro Cheesemaker II, Cheddarmaster Cheddaring Tower, and Stoelting Automatic Cheddaring Machine) showed less evidence of extensive cheddaring than Cheddar cheese manufactured by older conventional methods. However, in uniformity of body and closeness of texture, cheeses made by these automated systems were equal to or better than cheese cheddared in the traditional manner.
A MODEL SYSTEM FOR CURD FORMATION AND MELTING PROPERTIES OF CALCIUM CASEINATES.


A lipid-free model system was developed to aid in the study of melting and other functional properties of calcium caseinates in their application to imitation process cheese. The model system was formed by the controlled addition of water to an alcohol suspension of the protein. At a critical water concentration, a curd was formed spontaneously. The recovered curd was melted and the area of the melt determined (melt area index). The amount of water added, rate of water addition, and the source of caseinate affected the melt area index. A protein fraction, recovered from the supernatant, was rich in \(\beta\)-, \(\alpha\)-, and \(\kappa\)-caseins. The samples were also studied by light microscopy and SEM.

GELATINIZATION OF WHEAT STARCH. I. EXCESS-WATER SYSTEMS.


The surfactants sodium stearoyl lactylate (SSL) and monoglycerides inhibited swelling and solubility of wheat starch at temperatures below 95°C. At higher temperatures, SSL did not affect solubility, but monoglycerides effectively reduced solubility up to at least 120°C. SEM micrographs of starch heated in an amylograph also showed that SSL and monoglycerides kept starch granules intact at temperatures up to 85°C, whereas untreated starch was grossly deformed at that temperature. At 95°C the difference between the SSL-treated and the untreated starch had disappeared. Thus, SSL apparently is effective in retaining granule shape and starch solubility only at temperatures of 85°C or lower. Iodine affinity values, \(\beta\)-amylose limits, and gel filtration on Sepharose 28-CL columns all showed that amylose was leached from untreated starch at temperatures below 95°C. The surfactants effectively stopped the leaching of amylose.

GELATINIZATION OF WHEAT STARCH. III. COMPARISON BY DIFFERENTIAL SCANNING CALORIMETRY AND LIGHT MICROSCOPY.


A single endotherm was obtained at a high water-to-starch ratio (2:1). As the ratio was decreased to 1:1, the endotherm decreased and developed a trailing shoulder. Light micrographs of starches removed from the differential scanning calorimeter pans show that, at high water-to-starch ratios, birefringence is lost over a narrow temperature range of about 7°C. That range increases to about 30°C at a low water-to-starch ratio (1:2). No difference was found between endotherms of large and small granule wheat starches.

ROASTING OF NAVY BEANS (PHASEOLUS VULGARIS) BY PARTICLE-TO-PARTICLE HEAT TRANSFER.


A rotating chamber dry roaster using pre-heated ceramic beads as heat transfer media was used to roast navy beans. Processing conditions were: beads temperature, 240 and 270°C; bean-to-bead ratio, 1/10 and 1/15, and contact times of 1 and 2 min. Product temperatures achieved ranged from 92 to 125°C for the 8 runs. Heat transfer coefficients varied from 3.6 to 23.4 W/(m²°C). Roasted products showed reduced water-soluble nitrogen content and gel forming capacity, increased water-holding capacity, and cold paste viscosities, and no changes in available lysine and degree of starch damage. Residual trypsin inhibitor (TIA) and hemagglutinin activity varied from 92 to 22%, and 48 to 1%, respectively. A correlation was found to exist between nitrogen solubility index and TIA of products. Roasting caused fracture and separation of hulls, and facilitated their removal.

FREEZE-FRACTURE ULTRASTRUCTURE OF WHEAT FLOUR INGREDIENTS, DOUGH, AND BREAD.


The structures of isolated flour components of mixed doughs (containing several combinations of ingredients), of fermented doughs, and of bread crumb were examined by the freeze-fracture technique. Although the shapes of the small and large starch granules were unaltered in doughs, the gluten and the water-soluble structures appeared completely different in the complex-dough system. In general, water was distributed in three forms: 1. coating around starch granules and yeast cells, 2. droplets, and 3. large areas; all three changed with protein development. Protein development was followed from a protein network in a flour-water dough to a sheetlike protein in a complete dough (containing flour, water, yeast, salt, sugar, shortening, malt, and oxidant). Both compositional and physical (dough development) effects were indicated. A transition stage between the two structures appeared after sugar was added. Fermenting a flour-water-yeast-salt dough did not affect the protein network structure, but fermenting a complete dough altered the sheetlike protein to a fine network. In bread, regular dense-structured sheets were observed. In most doughs, protein-starch interaction was clearly visible; thin "pearl chains" or thin protein strands connected starch and protein. Those interactions intensified after fermentation. In bread crumb, protein and starch were tightly connected.

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Literature Abstracts
DETERMINATION OF STARCH GELATIZATION BY X-RAY DIFFRACTOMETRY.


The potential of X-ray diffractometry for quantitative determination of gelatinization of starch was evaluated. The method, which used a "built-in" internal standard approach, had a total coefficient of variation of 99.87%. The diffractometry method also correlated well with a standard chemical method. Both procedures showed that critical moisture contents of 45-47% for maize and 55% for pea were necessary for complete gelatinization of these starches. Results from SEM ran parallel to the chemical and X-ray data, indicating that both methods measure physical changes taking place during gelatinization.

STARCH DEGRADATION IN ENDOSPERMS OF BARLEY AND WHEAT KERNELS DURING INITIAL STAGES OF GERMINATION


During initial stages of germination, patterns of starch degradation in kernels of barley and durum wheat appeared to be similar as assessed by SEM. Degradation started at the ventral crease edge of the endosperm-embryo junction and moved along this junction to the dorsal edge. This suggests that the site of initial α-amylase synthesis in germinating cereal grains is the embryo and not the aleurone layer.

TEFLON AND NON-TEFLON LINED DIES: EFFECT ON SPAGHETTI QUALITY.


Six samples of semolina, milled from durum wheats of varying quality, were extruded through Teflon and non-Teflon lined dies. Extruded spaghetti was evaluated for color and cooking quality. The effect of cooking time on cooking quality was also evaluated. Teflon-extruded spaghetti had better appearance and cooking quality than its non-Teflon-extruded counterpart. Strong gluten, whether in the Teflon- or non-Teflon-extruded products, improved cooking quality and tolerance to overcooking. SEM of the outer surface of dry and cooked spaghetti elucidated, in part, the differences in appearance and cooking quality of the products extruded through both die forms.

USE OF MICROSCOPY IN THE STUDY OF VEGETABLE PROTEIN TEXTURIZATION.


A review with numerous references and SEM, TEM, and LM micrographs of texturized soy products.

DECORATING PEARL MILLET AND GRAIN SORGHUM IN A LABORATORY ABRASIVE MILL.


The decortication behavior of random mating populations of pearl millet and cultivars of grain sorghum was studied with Shepherd's modification of the Udy cyclone mill. Sorghum bran was removed in large flakes during decortication, and pearl millet bran was removed in smaller flakes. Neither sorghum nor millet was degemmed during decortication. Millets grown in Sudan required less time to decorticale than Kansas-grown millets. Fractionation of the decorticale and the decorcticated grain, using screens and a seed blower, indicated that differences in decortication rate were largely related to endosperm softness.

HARDNESS OF PEARL MILLET AND GRAIN SORGHUM.


The hardness of various populations of pearl millet and cultivars of grain sorghum was determined by particle size analysis after the grains were milled on attrition and roller mills. Millets grown in Sudan were, in general, softer than Kansas-grown ones. However, kernel vitreousness did not parallel grain hardness as determined by particle size analysis. Furthermore, tempering either millet or sorghum before milling shifted the particle distribution to larger sizes compared with those of nontempered samples.

MALT MODIFICATION ASSESSED BY HISTOCHEMISTRY, LIGHT MICROSCOPY, AND TRANSMISSION AND SCANNING ELECTRON MICROSCOPY.


Modification in a kilned malt sample was studied by a combination of histochemistry, LM, TEM, and SEM. Hydrolysis of cell walls, proteins, and starch was most extensive in the starchy endosperm area adjacent to the scutellar epithelium. Some hydrolysis occurred in areas adjacent to the aleurone layers; hydrolysis decreased as distance increased from the embryo end to the distal end and from the aleurone layer to the center of the starchy endosperm. While no rigid sequence of hydrolysis was observed, generally, cell-wall hydrolysis was more extensive than protein hydrolysis, and starch hydrolysis seemed to take place gradually in the late stages of malting and kilning. Small starch granules were hydrolyzed more extensively than large granules.
PHYSICAL AND CHEMICAL CHARACTERISTICS OF EXTRUDED RICE FLOUR AND RICE FLOUR FORTIFIED WITH SOYBEAN PROTEIN ISOLATE.


Texturized products were produced from rice flour and rice flour fortified with soy protein isolate (SPI) by extrusion cooking. Water absorption capacity (WA) of the extrudates was slightly depressed in the presence of SPI. SEM revealed that the fine structure of the textured products differed subtly from each other and that SPI formed fine strings in the starch matrix. Solubility tests indicated that thermoplastic extrusion processing increased noncovalent interaction and 55-bonding resulting in decreased protein solubility. 75 subunit of the soy protein seemed to be more affected than the llS subunit. Insolubilization was significantly less when rice flour and SPI were processed simultaneously indicating a sparing effect in their mutual presence.

ULTRASTRUCTURAL ANALYSIS OF A SOYBEAN PROTEIN ISOLATE.


Various fixation techniques were employed in an effort to prepare a dry protein isolate from soybeans for analysis using transmission electron microscopy. A number of fixation schemes proved successful, and different properties of the material were brought out by the various treatments. Comparison of electron micrographs of the isolate fixed and extracted in different ways yielded an extensive means for determining the distribution of fat and protein in the sample and the effect of moisture on that distribution. Analysis of the isolate demonstrated the occluding effect of dry protein for fat located in walls of the protein shells of the isolate.

FUNCTIONAL PROPERTIES OF THE GREAT NORTHERN BEAN (PHASEOLUS VULGARIS L.) PROTEINS, AMINO ACID COMPOSITION, IN VITRO DIGESTIBILITY, AND APPLICATION TO COOKIES.


The Great Northern bean flour proteins, albumins, and protein isolates were characterized by high acidic amino acid content while globulins and protein concentrates had high proportion of hydrophobic amino acids. Sulfur-containing amino acids and leucine were first and second limiting amino acids in these proteins, respectively. The bean proteins were resistant to in vitro enzymatic attack. Heating improved in vitro susceptibility of these bean proteins to enzymatic hydrolysis. Moist heat was more effective than dry heat in improving in vitro digestibility of the bean proteins. Cookie diameter was negatively correlated to alkaline water retention capacity of the blends of wheat flour with bean flour (r = -0.90) and protein concentrates (r = -0.93).

EFFECT OF HEATING TIME OF SOYBEAN ON VITAMIN B-6 AND FOLACIN RETENTION, TRYPsin INHIBITOR ACTIVITY, AND MICROSTRUCTURE CHANGES.


Heating treatments of boiling 20 min or autoclaving 5, 10, or 20 min, of soaked (25°C for 10 h) soybeans, significantly influenced vitamin B-6, free folacin, trypsin inhibitor activity, water absorption, moisture content, and blue, green, and amber color values in the cooked soybeans. Analysis of covariance showed a relationship (P ≤ 0.05) between water absorption after cooking with total folacin in cooked soybeans and water absorption after cooking with blue color values. Other relationships (P ≤ 0.05) were observed in cooked soybeans between texture and total folacin, moisture and trypsin inhibitor activity, and trypsin inhibitor activity and free folacin.

EFFECT OF LYE PEELING CONDITIONS ON SWEET POTATO TISSUE.


Heat penetration effects on sweet potato tissue resulting from 3 lye peeling treatments were evaluated using LM and SEM. Heat-mediated starch gelatinization, cell wall separation, chromoplast disruption, and enzymatic discoloration were observed in varying degrees according to the peeling treatment. Starch gelatinization, cell wall separation, and chromoplast disruption decreased in the order: 15-min peel; 30-min pre-soak, 6-min peel. Discoloration occurred in significant amounts only in the 6-min peel because heat penetration was sufficient to disrupt laccifier organization but was insufficient to inactivate the polyphenol oxidase enzyme. The 30-min pre-soak, 6-min peel treatment provided the most attractive finished product.

EFFECT OF pH ON EXTRACTABILITY OF CALCIUM AND OXALATE FROM ALFALFA LEAFLETS.


Alfalfa leaflets (structure in alfalfa with the highest Ca and oxalate concentrations) were ground and passed through a 0.7 mm screen. The ground material was extracted with 0.06 to 1.00N HCl by boiling for 20 min. The residues were washed, dried, and ashed in a low-temperature oxygen plasma asher. Ash was examined in wet mount by LM (normal and polarized light). Dried
Literature Abstracts

ash was coated with 10 nm C and examined for elemental maps (Ca, P, Si) and for relative element profiles. The specimens were subsequently coated with 15 nm Au-Pd and examined by SEM. Apparently, Ca oxide was not soluble under the conditions prevailing in the bovine digestive tract (20-33% of Ca in alfalfa is in the form of oxide).

MICROANALYTICAL QUALITY OF TOMATO PRODUCTS: JUICE, PASTE, PUREE, SAUCE AND SOUP.


A national retail market survey was conducted to determine the sanitary quality of the tomato products. The official methods of the Association of Official Analytical Chemists were used to count molds, rot fragments, fly eggs, and maggots. The most frequently encountered defect was mold decomposition. Howard mold counts for all products ranged from 0 to 57%. Mold count means ranged from 2.0 to 9.4%. The percent of samples containing mold ranged from 55.4% for tomato soup to 98.9% for tomato paste. Rot fragment counts ranged from 0 to 560/g, fly egg counts ranged from 0 to 5 and maggot counts ranged from 0 to 2/100 g-sample.

DEHYDRATED MAPLE SYRUP.


Selected grades of maple syrup (34% moisture) were dehydrated by 2 dissimilar methods. Differences in color and flavor of reconstituted dried products and the maple syrups from which they were derived were minimal. Moisture content, bulk density, hygroscopicity, and ease of reconstitution of the dehydrated products showed minimal divergence from corresponding values for the table sugar used as a standard. Microscopic examination showed very different structures for table sugar and each of the two dehydrated products. This study of some important physical characteristics indicates that either of the dehydrated products would provide the maple industry with a new and useful product. The lower moisture content, greater weight per unit volume, and method of continuous manufacture make the patent product preferable.

EFFECT OF FOUR ANTICAKING AGENTS ON THE BULK CHARACTERISTICS OF GROUND SUGAR.


Fine silicon oxide, sodium aluminum silicate, tricalcium phosphate, and calcium stearate powders were admixed with dry ground sugar at four concentration levels between 0.1 and 2%. Appreciable increase in loose bulk density and decrease in compressibility were noticeable at 0.1% concentration in all four agents. The effect reached an apparent peak or a flat maximum at an agent concentration of about 0.5-1.0%. With the exception of silicon oxide-treatec powders. The increase in density was accompanied by a corresponding decrease in compressibility. Bulk parameters (i.e. density and compressibility) were more sensitive indices to changes occurring in powders as compared to parameters determined in dehydrated products (i.e. yield in shear, internal friction, and relaxation patterns). Results are explained in terms of possible bed arrays and their scatter by differences in particle size and shape distribution. Support for these explanations is presented in SEM micrographs of sugar treated and untreated particles.

WATER-HOLDING CAPACITY AND TEXTURAL ACCEPTABILITY OF PRECOOKED, FROZEN, WHOLE-EGG OMELETS.


Precooked, frozen omelets were analyzed for moisture loss, expressible moisture, shear force, and sensory evaluation to determine water-holding capacity and textural acceptability. Addition of 0.1% xanthan gum, application of moist heat in cooking, and cryogenic freezing with liquid carbon dioxide or nitrogen minimized moisture loss and shear force. Sodium carboxymethylcellulose (CMC), pregelatinized tapioca starch, and sodium triply-phosphate additives performed satisfactorily, but omelets containing xanthan gum were consistently rated highest in sensory evaluation of several treatments, including fresh and untreated control omelets. Steaming omelets for 5 min combined with cryogenic freezing produced a desirable omelet, requiring no additives. Steamed omelets were rated comparable to baked omelets in most sensory parameters.

A STATISTICAL EVALUATION OF HISTOLOGICAL CHANGES CAUSED BY NONMEAT PROTEINS IN WIENER BATTERS.


Histological studies are usually illustrated with "representative" fields. Studies with uncooked wiener batters containing substantial amounts of nonmeat protein showed that the nonmeat protein prevented the formation of the typical fat and protein matrix that is characteristic of all-meat mixtures, but only in some microscopic fields of some slices. The two-within-four randomization test was used in conjunction with an appropriate sampling plan to determine the percentage of slices in which the ingredients were "clumped" rather than distributed randomly across a microscopic field. This converts histological results to a numerical score by which various formulations can be compared.
CALCIUM ACTIVATED NEUTRAL PROTEASE HYDROLYZES Z-DISC ACTIN.


Fractionation of myofibril proteins using Hasselebach-Schneider solution (HS) and a potassium iodide solution (KI) revealed that an unusual form of actin existed in the H-S and KI extracted myofibrils which could be easily hydrolyzed by calcium-activated neutral protease (CANP). That actin was similar in amino acid composition to normal thin-filament actin but differed in isoelectric pH, solubility in KI, and antigenicity in mice. Indirect immunofluorescence using antiserum mortem aging.

FURTHER STUDIES ON THE ROLES OF THE HEAD AND TAIL REGIONS OF THE MYOSIN MOLECULE IN HEAT-INDUCED GELATION.


Heat-induced gelation properties of 2 proteolytic fragments of myosin, heavy (HMM) and light meromyosin (LMM), were studied by rigidity measurement in a band type viscometer and by a direct examination using a scanning electron microscope. A heat-induced network-forming ability for both LMM and HMM was found in 0.6 M KCl at a pH 6.0. LMM produced gels corresponding to a reversible helix-coil transition at temperatures ranging from 40 to 70°C, with little evidence of aggregation as assessed from a turbidity change of the system. Contrary, HMM associated irreversibly producing a gel with increased rigidity at pH 5.0 and a salt concentration of 0.1 M. Oxidation of SH-groups appeared to be involved only in HMM and not in LMM gelation processes.

ULTRASTRUCTURAL CHANGES DURING CHOPPING AND COOKING OF A FRANKFURTER BATTER.


Bacterial colonies were grown on the "Würze" type of Millipore membrane filter, fixed in Karnovsky solution, postfixed with OsO4, dehydrated in a graded ethanol series, and critical-point dried. The colonies were observed in relation to the potential application of this process as one way of upgrading fish waste and under-utilised species. The colonies were also examined by SEM.

EFFECT OF WATER IMMERSION ON THE MICROTOPOGRAPHY OF THE SKIN OF CHICKEN CARCASSES.


Water-immersion cleaning and chilling of poultry carcasses causes the skin to swell and exposed deep channels and crevices in the skin surface as a result of water uptake. These changes were demonstrated by EM and were shown to be dependent on the time of immersion and the temperature of the water. Addition of NaCl to the immersion medium and manipulation of the medium pH did not markedly affect or prevent these changes. The implications of these changes for microbial contamination during water-immersion chilling are discussed.
THE ULTRASTRUCTURE OF GERMINATING BARLEY SEEDS.  
I. CHANGES IN THE SCUTELLUM AND THE ALEURONE LAYER IN NORDAL BARLEY.


The ultrastructure of the scutellum and the aleurone layer has been examined in Nordal barley following malting for 0, 30, 72, and 162 h at 15°C. Thick sections from the seeds were stained with Calcofluor White M2R New and examined in the LM to determine the extent of cell wall degradation in the endosperm. Seeds which showed in the incident UV-light a modification closest to the average, were selected for TEM. It is concluded that the scutellar epithelium provides the enzymes during the initial degradation of the endosperm. In seeds malted for 162 h, aleurone cells with a cytoplasmic organization indicative of active metabolism are located in the embryo part of the seed, whereas aleurone cells adjacent to unmodified endosperm in the distal end of the same seed show little or no structural sign of activity.

THE ULTRASTRUCTURE OF GERMINATING BARLEY SEEDS.  
II. BREAKDOWN OF STARCH GRANULES AND CELL WALLS OF THE ENDOSPERM IN THREE BARLEY VARIETIES.


The breakdown during malting of starch granules and cell walls of the endosperm, as well as the morphological changes occurring in the scutellum and the aleurone layer, have been examined by thin-sectioning and freeze-fracturing in the barley varieties Nordal, Minerva, and Klages. Intact starchy endosperm cells contain large and small starch granules embedded in a matrix. In most cases the fracture plane exposes the smooth surface of the starch granules and occasionally adhering material, which probably consists of remnants of the amylloplast envelope. Degradation of the endosperm tissue during malting starts at the embryo end, and the transitional zone between intact and degraded endosperm is approx. one cell layer wide. The degradation of the large starch granules affects at the beginning only their outer edge. Subsequently, corroding channels form toward the center of the granules and elicit a rapid radial direction. The 3 barley varieties examined differed in melting quality, but the differences were not reflected qualitatively in the ultrastructure of the scutellum, aleurone layer, or the endosperm during malting.

EFFECT OF MONOGLYCERIDE ON SOME REHYDRATION PROPERTIES OF POTATO GRANULES.


Monglyceride incorporation in an Add-Back (A-B) or Freeze-Thaw (F-T) process decreases the potato granules' Blue Value Index (BVI), Swelling Power (SP), rehydration rate, and Water-Binding Capacity (WBC), and increases the intact sound cell count. With 0.2% monoglyceride, SP, WBC, and rehydration rate of A-B granules were 8.4, 6.7, and 6.4%, resp., lower than F-T granules, while the BVI of A-B granules was 25.3% higher. Monglyceride increase above 0.2% caused negligible changes. The intact sound cell count was higher when a precooking step was applied. The porous F-T and compact A-B granule structures partially accounted for differing water penetration rates. X-ray analysis revealed a weak monoglyceride-starch interaction during processing.

COMPARISON OF THE CRYSTALLINITIES OF WHEAT STARCHES WITH DIFFERENT SWELLING CAPACITIES.


A comparison has been made of the degree of crystallinity in a number of varieties of wheat starch with different swelling capacities. High swelling capacities are associated with relatively disordered arrangements of polymer within granules. Work on fractionated starches confirms the small granules tend to be more crystalline than large granules. However, small granules of wheat starch have greater swelling capacities than large granules under the conditions of paste formation. This may be because the small starch particles contain less lipid than the large granules. Although differences in crystallinity have been recorded between different wheat varieties, it is stressed that they may not be primarily responsible for the observed differences in swelling.

CHARACTERIZATION OF STARCH FROM GINGER ROOT (ZINGIBER OFFICINALE).


Starch was isolated from ginger root in 12.3% yield. The starch that contained 22.2% amylose had a density of 1.517 g/cm3 and an A type x-ray diffraction pattern. Solubility studies revealed low swelling power and solubility in water, and reduced solubility in dimethylsulfoxide (DMSO) and in KOH, suggesting homogeneous and strong bonding forces maintaining the granular matrix. Information from the Brabender amylograph indicated a relatively high initial pasting temperature (80°C) and resistance to mechanical shear upon gelatinization, resembling starches modified by cross-linking. For LM, starch granules were suspended in water, a 2% KI solution, and anhydrous DMSO.
SCANNING ELECTRON MICROSCOPY OF ENZYME DIGESTED VARAGU STARCH GRANULES.


Amyloytic susceptibility of native varagu starch granules was followed chemically and microscopically. SEM revealed that the attack by salivary α-amylase resulted in a gradual erosion of the surface followed by granule penetration at certain locations. Attack by glucoamylase, on the other hand, was more uniform and resulted in pitting and formation of depressions all over the surface.

KRITISCHE UNTERSUCHUNGEN ÜBER BILDUNG UND ENTWICKLUNG DER TRITICEEAN-STÄRKEKÖRNER. [Critical examination of the development of Triticeae starch grains].


At the right time when the first anthers appear at the top of the ears, large quantities of pointed starch grains are seen in the young ovary. The diameters of the small starch grains were measured there until the milky maturity stage was reached. In this way the identity and the growth of the small starch grains had been confirmed. After several days, when the diameters of the small starch grains reached 4 to 6 μm, the second stage of development began. The starch grains were progressively surrounded with a structure which resembled two lips with a furrow between them. When the two lips surrounded the "nucleus", the thickness and the diameter of the granule continued to increase in such a way that the furrow became shallower and was present in the mature biconvex structure only as a shallow score.

LOCATION OF αs1, β- AND κ-CASEIN IN ARTIFICIAL CASEIN MICELLES.


Artificial casein micelles were prepared from mixtures of αs1, β-, and κ-casein, in which one of these components had been labelled with small gold beads: 250 mg protein to be labelled was dissolved in 7 mL water and heated to 100°C; a solution (5.25 mL) containing 1.0 g ascorbic acid, 1.5 g NaOH, and 75 mg KCl/100 mL was added and the mixture was heated again. Then 5.25 mL of a solution containing 10 g HAuCl4/100 mL was slowly added with vigorous stirring, after which the solution was cooled to room temperature, adjusted to pH 9 with 0.5 N HCl, and dialyzed against distilled water for 16 h. Labelled artificial micelles were immobilized in agar gel microcapsules, fixed with 1% OsO4 for 16 h, embedded in a resin, sectioned (50 μm), and examined by EM without additional staining. It was demonstrated that κ-casein was located at the micellar surface, whereas αs1 and β-casein were scattered over the entire micelle.

EPOXY RESINS ARE MUTAGENIC: IMPLICATIONS FOR ELECTRON MICROSCOPISTS.


Epoxides, as highly reactive alkylating agents, must be suspect as mutagens. Using the Salmonella bacterial test system (the Ames test), we have confirmed the mutagenicity of 13 epoxy resins used as tissue-embedding agents in TEM: Quetol 651, DER 732, DER 736, vinyl cyclohexene dioxide (ERL 4206), diglycidyl ether, Epon 812, Epon 828, Polybed 812, Effapoxy, and Araldite RD-2, 502, 506, and 6005. While of this group only vinyl cyclohexene dioxide is a documented animal carcinogen, the other resins have levels of mutagenicity in the same range as known epoxide carcinogens; some may be weak carcinogens which have escaped detection in animal tests. We conclude that epoxy resins should be labeled and handled as mutagens and suspect carcinogens. We discuss some factors affecting possible hazards to laboratory workers, and present a protocol for minimizing inhalation and skin contact during resin use. (Copyright 1982 by Academic Press, Inc., 0006-2944/82/050144-13502 00/0).
Each paper in this volume contains a Discussion with Reviewers. This discussion follows the text and should be read with the paper. Each paper submitted to SEM, Inc. for publication is reviewed by at least three, up to an average of five, reviewers. The reviewers are asked to separate their comments from their questions. The comments are useful in determining the acceptability of the papers as submitted. Although the comments require no written response, in several cases, the authors have included responses to comments, or to questions phrased from, or based on, comments (either as a result of editorial suggestions or on the author's own initiative). Based on these comments approximately 15% of the submitted papers were not accepted for publication; while almost all of the others were asked to make changes involving from minor to major revisions.

The questions, for the most part, originate as a result of statements included in our cover letter accompanying each paper sent to the reviewers. The reviewers are asked to suppose they are attendees at a conference where this paper, as written, is being presented, and then ask relevant questions which would occur to them resulting from the presentation. From the questions so asked, some are not included with the published paper because the authors attended to them by text revisions. In some cases, editorial and/or space considerations may exclude inclusion of all questions asked by reviewers. The authors are asked to prepare their Discussion with Reviewers section in a camera-ready format. In some instances the authors edit the questions and/or combine several similar questions from different reviewers to provide one answer.

While all efforts are made to check that the questions in the printed version faithfully follow the views of the specific reviewer, the editors apologize, if in some instances, the actual meaning and/or emphasis may have been changed by the author.

The cover letter to the reviewers states:

"1. Your name will be conveyed to the author with your review UNLESS YOU ASK US NOT TO.

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In all cases sincere efforts are made to respect the reviewer's wishes to remain anonymous; however, in nearly 95% of the cases, the reviewers have given permission to be identified; so their names are conveyed to the authors and are included with the questions printed with each paper. An overall list of reviewers is provided in the opening pages of each SEM part. We apologize for any error/omissions which may occur.

Finally, readers are urged to be cautious regarding the weight they attach to the authors' replies, since the answers to the questions represent the authors' unchallenged views--except for minor editorial changes--the authors generally have the last word. Also, please consider that the questions were, in most cases, relevant to the originally submitted paper, and they may not have the same significance for the revised paper published in this volume.

If you disagree with the results, conclusions or approaches in a paper, please send your comments, as a Letter to Editor, typed in a column format (each column is 4-1/8 inches wide and 11-1/2 inches long; i.e., 10.5 by 29.3 cm.). Your comments along with author's response will be published in a subsequent issue.

The editors gratefully thank the authors and reviewers (see p. 248-249) for their contributions, invite your comments on ways to improve this procedure and seek qualified volunteers to assist with reviewing papers in the future.

ERRATA: Despite the best efforts of authors, reviewers and editors, errors may remain. Please help by pointing out errors that you notice. Please provide enough information to locate each error (volume, page, column, line, etc.) and indicate suitable correction.

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The help of the following reviewers for papers published in the first volume of FOOD MICROSTRUCTURE is gratefully acknowledged.

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Important Note: The time restrictions we work under require that each reviewer returns his review (along with the manuscript sent) within a set time from its receipt. Please do not commit yourself if you feel that you cannot respond within this time frame; while we are grateful for your desire and efforts to help us, the reviewers who do not respond in time, in fact, seriously hamper our efforts.

CONTACT: Om Johari, SEM Inc., P.O. Box 66507, AMF O'Hare, IL 60666, USA
Phone: 312-529-6677.
This not-for-profit organization was established with the following goals: a. Promotion of advancement of science of SEM and related material characterization techniques;
bi. Promotion of application of these techniques in existing and new areas of applications; c. Promotion of these techniques so that their users obtain the best information of the highest quality from their instruments.

The efforts of the organization are directed towards fulfillment of these goals. Suggestions on activities which SEM may sponsor are invited, and may be communicated to any one of the following persons:

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1983 MEETINGS SPONSORED BY SEM, Inc.

(i) The annual SEM meeting (called Scanning Electron Microscopy/1983) will take place during April 17-22, 1983, at Hyatt Regency Hotel, in Dearborn, Michigan. Dearborn is a historic town just outside Detroit and is home of several museums (including the famous Henry Ford Museum and Greenfield Village). Hyatt Hotel is connected by a frequent and free monorail service with one of the largest indoor shopping centers (Fairlane Town Center).

SEM 1983 meetings highlights will include a comprehensive equipment exhibition where almost all suppliers of SEM, related analytical equipment, and other suppliers/services will be in attendance.

Several tutorials on various basic aspects of SEM of interest to newcomers in scanning electron microscopy will be planned as a part of the SEM/1983 program.

A general session, several programs of common interest, and many specialized programs in Physical and Biological Sciences will be planned. Many of the programs planned for past SEM meetings will be repeated, several new programs may be planned. Persons interested in either organizing specific programs or seeing specific themes covered at SEM/1983 should contact Dr. Om Johari.

A registration form for SEM/1983 is provided below.

(ii) 2nd Pfefferkorn Conference on “The Science of Biological Specimen Preparation for Microscopy and Microanalysis.”

Immediately following the SEM/1983 meetings, a special program on biological specimen preparation will take place from April 23-28 at the Sugar Loaf mountain resort located near Traverse City (in the scenic area of northwestern Michigan, about 240 miles from Detroit). The emphasis at this program will be on the Science of specimen preparation (and not on recipes and tips). Preparation of specimens for all types of microscopy (light optical, SEM, TEM, STEM) and microanalysis (x-ray, LAMMA, etc.) will be covered.

The organizers of this special conference are Prof. J.P. Revel (Div. Biology, 156-29 Cal Tech, Pasadena, CA 91125; 213-356-4986 as the general chairman); Dr. T. Barnard, Oslo Univ., Norway (02-456191), Dr. G.H. Haggis, Agriculture Canada, Ottawa (613-995-3700 x219), and Prof. T. Murakami, Okayama Univ., Japan (0862-23-7151 x425). Many invited contributions will be planned. Interested contributors are urged to contact one of the organizers. Attendance at this conference will be by application or invitation only. Further details are now available.

REGISTRATION FORM FOR SEM/1983

Attendance at all tutorials, sessions and exhibitions will be by registration only. Registration fees are not refundable and registration will not be transferable.

Date Payment Received Registration fee
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PAPERS IN PROCESS FOR PUBLICATION IN FUTURE ISSUES OF FOOD MICROSTRUCTURE

Offers of following papers have been received for possible publication in the future issues of this journal.

Methods to Study the Structure of Lipids in Food Systems
G.G. JEWELL, Cadbury Schweppes Ltd., Univ. Reading, U.K.

Comparison of Two Techniques for Preparation of Fat Crystals for Scanning Electron Microscopy
A.K. SMITH and S.F. LEE, Univ. Guelph, Ontario, Canada

V.E. COLOMBO, F. Hoffmann-La Roche & Co., Basel, Switzerland

Processing of French Fries as Elucidated by EM Microscopy
D. HADZIYEV, et al., Univ. Alberta, Canada

Ultrastructure Studies of Pasta
P. RESMINI and M.A. PAGANI, Inst. Industrie Agrarie, Milano, Italy

Size Threshold for Sensory Detection of Graininess in Canola Based Products
B.K. VANE and M. VAISEY-GENSER, Univ. Manitoba, Winnipeg, Canada

The Structure of Fresh and Desiccated Coconut
J.F. HEATHCOCK and J.A. CHAPMAN, Cadbury Schweppes Ltd., Univ. Reading, U.K.

Microstructure of Spices and Spice Products - Present Status and Future Scope
J.S. PRUTHI, Krishna Consultants, Ludhiana, India

Morphometry and Stereology of Meat: Data Collection by Scanning Light Microscopy
H.J. SWATLAND, Univ. Guelph, Ontario, Canada

A Review of the Muscle Cytoskeleton and Its Relation to Texture
D.W. STANLEY, Univ. Guelph, Ontario, Canada

Statistical Evaluation of Histological Changes in Wiener Batters During Chopping and Cooking
A.G. KEMPTON, Univ. Waterloo, Ontario, Canada

Pre-rigor Pressurization Effects on the Calcium Activated Factor and Other Proteases from Bovine Muscles
E.A. ELGASIM, Oregon State Univ., Corvallis

Field Spectroscopy in the Food Production Chain
E. BRACH, Agriculture Canada, Ottawa, Canada

Microstructure of Microalgal Health Foods
L.-P. LIN, National Taiwan Univ., Taipei, China
FOOD MICROSTRUCTURE will be the subject of a program scheduled for April 20-22, 1983 at the Hyatt Regency Hotel, in Dearborn, Michigan. This is a continuation of a successful series which was started in 1979 (see page v, and outside back cover of the first issue, and outside back cover of this issue for an example of the types of papers suitable for these meetings).

All types of foods, including vegetables, grains, sea foods, meat, dairy products and others will be covered. Papers on the fundamental aspects of food microstructure such as the molecular and colloidal forces which determine it, and the practical relationship between food microstructure and processing, ingredient changes, shelf life, consumer acceptability, and other food-related areas are invited. Papers may be in the form of tutorials, reviews or original contributions (see inside back cover). Techniques may include transmission electron microscopy, scanning electron microscopy, light microscopy, x-ray microanalysis or related microscopy/microanalytical methods.

Papers offered for this program will be published in the semi-annual Journal, "FOOD MICROSTRUCTURE". The organizers of the program and the editors of the Journal are Dr. S. H. Cohen, U.S. Army R&D Command-Food Research Laboratory, Natick, Mass. (Telephone 617-651-4578); Prof. E. A. Davis, Food Science Department, University of Minnesota, St. Paul (Telephone 612-373-1158); Dr. D. N. Holcomb, Kraft R&D, Glenview, IL (Telephone 312-998-3724); and Dr. M. Kalab, Food Research Institute, Agriculture Canada, Ottawa (Telephone 613-995-3700 Ext. 272).

The Food Microstructure program in Dearborn will be held in conjunction with the SCANNING ELECTRON MICROSCOPY/1983 meetings from April 17-22, 1983. Registrants at the Food Microstructure program will be able to attend all of the activities of the SEM meeting at no additional charge. Of particular interest should be: several tutorials, and programs on Analytical Electron Microscopy (including STEM), Microprobe Surface Analytical Techniques, Particulate Characterization, Plants, and many areas of physical, biological and biomedical application. A comprehensive equipment exhibition will take place during April 19-22, 1983. Complete details of SEM meetings are available on request.

The registration fee for this program is $40.00, if paid before Jan. 31, 1983, and $55.00 after that. For subscription to the journal include an additional $50.00 (for U.S. delivery) and $55.00 for elsewhere.

A Call for Papers, Registration Form, details of travel support and Hotel information are available on request. For more information on this program and the Journal, "Food Microstructure" contact Dr. Om Johari or one of the organizers.
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INSTRUCTIONS TO AUTHORS

FOOD MICROSTRUCTURE

Papers for publication in Food Microstructure can be offered at any time. Papers intended for oral presentation at the Annual Food Microstructure meeting in April are due January 15th. Only papers acceptable for publication are allowed oral presentation.

In a letter accompanying the paper, authors must provide names and complete addresses of at least four persons competent to review their paper. Please note: a. Suggested reviewers must neither be from authors current or recent affiliations, nor coworkers; b. preferably suggested reviewers should be critical reviewers in the field (e.g., whose work is being extensively referred); and c. Authors are not expected to personally know nor required to contact the suggested reviewers. From the names suggested by the authors and SEM's editors, reviewers will select the most suitable reviewers irrespective of their geographical location. Each paper will be intensely reviewed by at least three, and often more, reviewers.

The initial paper (hereafter referred to as a ‘paper’) should conform to these Instructions. However, to be published after reviewing, the final manuscript (hereafter referred to as a ‘manuscript’), must be submitted on model sheets conforming to the Manuscript Submission Guidelines; these guidelines and model sheets (a sample model sheet is available on request) will be mailed along with the reviewers’ comments. In addition to all the text, the manuscript must also contain author’s publishable responses to questions raised by the paper’s reviewers. (See, e.g., discussion with reviewers in this issue.)

Three types of papers can be offered. The authors must indicate type of paper and carefully adhere to the applicable definition, since the reviewers and editors judge the paper accordingly:

CONTRIBUTED PAPER: Presents new unpublished findings.

REVIEW PAPER: Includes an extended literature review and complete bibliography, emphasizes author’s new unpublished findings and in an extended discussion puts the topic in proper perspective.

TUTORIAL PAPER: Contains an organized comprehensive review and bibliography of all relevant published material as for a teaching lecture.

INSTRUCTIONS FOR SUBMISSION OF PAPERS

Type paper in double-spaced format on 8½ x 11 inch (or similar size) paper. A length limit is not imposed on papers. Short, but complete, papers are welcome.

The paper should include title page, abstract, all headings and text. On the title page include a short title which accurately reflects the contents of the paper; an informative running head consisting of no more than 50 characters, including spaces between words; names and affiliations of all authors; name and complete work and home address and phone numbers of the person to contact, and 10 key words/phrases suitable for subject index. For review papers the title page must indicate page numbers containing new material (e.g. “new material will be found on pages 1-10”).

An Abstract (of 100-250 words) is required for all papers. The abstract should be a concise statement of the purpose of the paper and of the major results obtained. It must be self-explanatory, and free of phrases such as “will be described”, “is discussed”, “are presented”, etc.

The Introduction to the paper must contain a clear, concise statement of the purpose of the paper and the relationship of this paper to what is already in the literature. As applicable, a Materials and Methods section, with complete specimen preparation information must be included so that the work can be duplicated by others.

Equations should be numbered consecutively, using arabic numerals. Each symbol and abbreviation should be defined when first used. SI (metric) units must be used. U.S. customary (English) or other metric units, if used, must be given in parentheses.

REFERENCES

Include all references relevant to paper. Do not use too many of your own references. References can be made only to readily available published work and to papers in press. Work in progress, manuscripts in preparation, manuscripts submitted, unpublished experiments, and personal communications, must be excluded from the reference list, but may be acknowledged in the text (in parentheses).

The reference list at the end of the paper must be organized in alphabetical order by the first authors’ names. Full titles of papers and inclusive pagination must be included.

In the text, cite references in one of the following two styles:

a. Cowley (1967) or (Cowley, 1967) or Crewe and Wall (1970). If there are three or more authors, use the form Venables et al. (1978). If more than one paper is published in the same year by the same author (or group of authors) use the form (Rose, 1974a), etc.

b. As long as there is consistency, either superscript1 or full size numerals in brackets [1] can be used. In this case, the numbering must be in sequence in the reference list, but the references will generally not appear in sequence in the text.

Examples of acceptable reference formats are:


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Number each figure and table with an arabic numeral and refer to them in sequence in the text. Several illustrations within a figure must be designated a, b, c, etc. Each table must have a title. Each figure must have a caption—either on its own page or all captions should be placed together on separate pages. Very important: Use arrows or letters to identify features referred to and so indicate in the caption. Illustrate text with the fewest photographs possible. Indicate magnification on photos by a line of, e.g., 1μm, 10μm, 100μm, or 1 mm length; identify either on the photo or in the caption. Use μm, μm, or mm, not μ, μ, or μ.

Quality of Illustrations. Photographs should be clear, clean, unscreened, (screened photos are not acceptable), black and white glossy prints. Color photographs can be published by prior arrangement between the author and the managing editor, whereby the author will be asked to pay the additional cost.

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For submission of papers and inquiries contact: one of the editors, or Dr. Om Johari, managing editor, (phone 312-250-6677), P.O. Box 66507, AMF O’Hare, IL 60666 USA. (Street address, if needed is: 1034 Alabama Dr., Elk Grove Village, IL 60007, USA).

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