

APPLICATION OF THE FREEZE-DRYING TECHNIQUE
FOR MICROSCOPIC STUDY OF
FAT IN CAKE BATTER

by

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CHAPTER I

INTRODUCTION

One of the chief tools in microscopic study of any material is a means of dehydrating the material in such a way that its true structure is maintained. In the study of batters and doughs, this has not been accomplished to the satisfaction of those working in the field (8, p. 197), (40, p. 44). The most frequently used procedure for studying such materials has been that of compressing and spreading a drop of batter between a microscope slide and a cover glass (9, p. 189), (25, p. 186), (20, p. 242). The possible physical changes brought about by this compression have often been ignored. The solutions normally employed as dehydrating agents are known to cause shrinkage of starch and protein, making them unusable for the study of batters and doughs (40, p. 44).

The freeze-drying technique has been recognized by increasing numbers of research persons annually as a prerequisite to cyto- and histochemical research (22, p. 33). Freeze-drying of animal tissue has been routine laboratory procedure for a number of years. Recently, modifications and improvements in the equipment (21, p. 572) have made

the freeze-drying of plant tissue, heretofore virtually impossible, a practical method (22, p. 36). Is it not feasible that this method of dehydration might be adapted to the microscopic study of batters and doughs? Artifact-free dehydration could make possible the preparation of permanent slides, so valuable to adequate microscopic study. Cross sections of batters could be studied exactly as can the baked products, thereby eliminating problems encountered when results attained by different methods are compared.

A cake batter is a complex system, consisting of such basic ingredients as flour, milk, fat, sugar, salt and eggs, many or all of which might be included. Prior to mixing, many of these ingredients are pretreated, thereby intentionally or inadvertently altering the mixing properties. The fat is usually hydrogenated, the flour bleached and the milk pasteurized and homogenized or dried for use in a mix. Additives may be incorporated for purposes of changing physical properties of these ingredients or increasing the nutritive value. The final structural organization of the batter is based not only on the ingredients used and the treatments they have received, but upon the manner in which they are combined. Without recipe adjustment, a single-stage mix or "quick cake" is inferior to one prepared by the conventional method. This

is no doubt a structural defect caused by manipulation.

Research could be conducted to determine the necessity for additional ingredient alteration if improved methods of microscopic study were available. An example of what can be accomplished is the finding that added emulsifiers produce a more even dispersion of fat in the batter and consequently a better cake. These conclusions were based partially upon the method of study whereby fresh batter is compressed under a cover glass (9, p. 189), (25, p. 186), (20, p. 242). What might be possible if more adequate microscopic methods were made available to those interested in the study of batters and doughs?

Numerous applications of such a method might be made. In this day of preprepared foods, a ready-to-bake frozen batter would certainly be welcomed by the food industry and by the homemaker. At this time, frozen batters have not produced cakes of high quality. In a study by Mackey, Jones, and Dunn (33, p. 222) cakes baked from batters stored at -18°C . (0°F .) up to three months were smaller in volume than those baked immediately, while those stored at -35°C . (-31°F .) for the same time tended to develop irregularities in texture. In a further study, Mackey examined batter microscopically, using the smear method, and found that the appearance of frozen cake batter differed from the freshly prepared. The characteristic

air bubbles embedded in the fat particles appeared to coalesce and the fat tended to pull away from the aqueous phase (32, p. 263). If portions of batter could be successfully dehydrated, and the fat fixed, cross sections could be studied. By seeing the relationship of ingredients to each other, one might gain a better understanding of the effect of freezing upon the structure. This might in turn serve to promote more research leading toward the production of better cakes from frozen batters. This is an example of the many ways such a method might contribute to the production of better baked products by serving as a tool for studying the ingredients and their relation to each other.

CHAPTER II

REVIEW OF LITERATURE

Existing Methods for Microscopic Study of Batter and Dough Structure

A number of methods and materials have been used for microscopic examination of batters and doughs. Each has contributed toward the determination of physical structure and the relationship of the constituents to each other. Carlin (9, p. 190) studied the behavior of fats in cake batters by dropping a minute portion of the batter onto a slide and, with a cover glass, applying sufficient pressure to spread the batter to a thinness which would allow microscopic examination. This has been the most commonly accepted method of studying such material (9, p. 189) (25, p. 186) (20, p. 242).

Burhans and Clapp (8, p. 197) realized that such mechanical manipulation changes the microscopic picture. Examination of frozen sections was considered, but this was impractical because of the thickness with which frozen sections must be cut. To be viewed under high magnification, sections must be 10 microns or less in thickness. They froze bread dough with carbon dioxide, fixed and stained the fat with osmic acid vapor, dehydrated with calcium oxide, embedded the samples with paraffin and

sectioned them with a sliding microtome. Using this method, they were able to microscopically examine bread dough for changes due to mixing, processing, and baking. Studies of shortening distribution, leavening, and crumb qualities as correlated with the microscopic structure were possible with this method (8, p. 210).

Recognizing that dehydrating agents cause shrinkage of starch and protein which causes breaks between starch granules and protein, Sandstedt and co-workers (40, p. 44) embedded bread dough in plastic, ground these samples to expose the edges of dough films, and viewed them microscopically by reflected light. By employing this method, background confusion was eliminated; only the exposed cut surface being visible.

Structure of Batters and Doughs

A batter or a dough is a complex mixture containing water and flour as basic constituents. Thus gluten protein and starch are always present. Other ingredients which may or may not be present are fat, with or without added emulsifiers, milk and egg which contribute additional protein and emulsifiers, sugar, and salts. Lowe (30, p. 446) says that the texture of the finished product is determined by the ingredients, the proportion of the ingredients, the kind and amount of baking powder, the

extent of mixing, the temperature of the ingredients when mixed, and the method of mixing or combining ingredients.

Cake Batter

Workers are agreed that a cake batter is a colloidal system combining air with a fat and liquid emulsion. However, whether the fat is the dispersing medium or the dispersed phase is a matter which has concerned many. After microscopic examination, Morris (36, p. 62) speculated that air cells are surrounded by a film of sugar syrup or egg protein, or both, which is emulsified in fat. Grewe stated that a creamed mixture of fat, sugar, and eggs produces a water-in-fat emulsion (17, p. 804).

More recent investigations indicate that aerated fat is emulsified in the liquid medium. Carlin agreed with Grewe that a creamed mixture of fat, sugar, and eggs produces a water-in-fat emulsion, but he further stated that the emulsion is broken or reversed by the addition of flour (9, p. 191). Bailey and McKinney observed that the air appears exclusively in the fat and they reported that a batter can contain only as much air as the fat is capable of holding (5, p. 120). Carlin (9, p. 198) stated that air spaces in layer and pound cake batters are invariably surrounded by fat. These clusters appear to be distributed in a medium of flour and water in which are

dissolved sugar and salts. Liquid is not emulsified in the fat. The prominent feature, as observed by Mackey (32, p. 261), is clustering of closely-packed small air bubbles within fat particles. Greethead felt that films of fat formed in the batter prevented gluten from binding into a network structure during baking (16, p. 247). The degree of dispersion of fat has been shown to depend, to a great extent, upon the method, temperature, speed of mixing, and upon the type of fat employed, and the presence of emulsifiers (30, p. 475).

The dispersion of fat in the batter may be greatly altered by the addition of commercial emulsifiers, usually mono- or diglycerides. Jooste and Mackey (25, p. 187) noted that in cakes made with butter or hydrogenated shortening without an added emulsifier the fat appears in clumps, with the gas bubbles irregularly grouped. With the addition of three and six per cent glycerol monostearate, the fat is well dispersed and the gas bubbles evenly distributed. With the added emulsifier, cake volume is increased and cell structure is improved. In general, the addition of the emulsifier tends to improve cell structure, lightness and tenderness of the baked cake, but it does not significantly improve springiness, moistness, velvetiness and flavor (25, p. 194). Among others who have investigated their benefits, Carlin also found that

monoglyceride type emulsifying agents produce a finer dispersion of fat throughout the cake batter (9, p. 198).

The larger volume obtained when the emulsifier was present could not be correlated with incorporation of air or moisture retention. Apparently better gas retention during the latter stages of baking accompanied with greater elasticity of air bubble surfaces was responsible for the volume attained (25, p. 190).

In contrast to observations previously cited, which suggest that the air is incorporated and held in the fat, there is evidence that the presence of fat is not necessary for the aeration of a batter. When a small percentage of an emulsifier is included in a batter that contains no fat, an exceptionally high degree of aeration and dispersion of air bubbles can be produced. Neither angel food nor sponge cake contain fat, yet the batters have a high degree of aeration. In batters of this type, the high dispersion of air bubbles is stabilized by protein, and emulsifying agents when present (45, p. 66).

Reports dealing with the appearance of protein in cake batter are virtually lacking in the literature. Lowe (30, p. 447) says that gluten in flour forms a continuous network in which starch is embedded in baked products. In her microscopic examination of baked cake, Morr found no relationship between protein and fat distribution (35, p.80).

Bread Dough

When first mixed, there are no gas bubbles present in bread dough and there is no organized structure (8, p. 201). The structure of this dough upon developing is found to be essentially a continuous network of gluten. This is explained by the fact that the dough protein absorbs about 200 times its weight of water, and swells. On absorbing water it acquires adhesiveness and by mixing and kneading the three-dimensional network is formed. The fat is distributed on the surfaces of the gluten, the sugar and salt are dissolved in the free water, and the starch is distributed throughout the dough (30, p. 412-413). Burhans and Clapp noted that the shortening was dispersed upon the starch and protein interfaces until the dough was baked, when the fat coalesced into droplets (8, p. 213). Baker believes that all starch granules are embedded in the protein because a film of dough surrounding a gas bubble will not show a blue stain with iodine until the surface is scratched, apparently exposing the previously shielded starch granule (6, p. 36). Sandstedt and co-workers found this relationship upon microscopic examination of bread dough. They feel that a strong bond exists between starch and protein, which is due to a water deficiency (40, p. 48). The gluten wall of the dough cell gives it its gas

holding property (6, p. 41). As the gas cells expand with an increase in temperature during baking, the starch granules in the cell wall are pulled parallel to the film surface. This orients the granules prior to gelatinization. As the granules gelatinize they become soft and flexible, allowing them to become elongated and stretched, thus allowing the film of protein in which they are embedded to expand (40, p. 47).

Pastry and Biscuit Dough

Cooky dough was examined microscopically by Platt and Fleming by spreading the relatively dry dough on a microscope slide and placing a cover glass over this to aid examination. They found a structure in which fat existed in thin layers extending between or enveloping most of the starch granules (38, p. 392).

Lowe (30, p. 537) describes the fat in pastry and biscuits as being blended with the flour so that it coats or is adsorbed by the flour. Colored fat used in preparing biscuits can be seen distributed in layers or pools and adsorbed on the flour particles. There seems to be little tendency for the fat to form an oil-in-water emulsion because the proportion of liquid to flour is small and no egg is present (30, p. 458).

The Effect of Stress upon Constituents of Batters and Doughs

There are a number of external forces which alter the physical appearance of the various components of batters and doughs. Among these are hydration, dehydration, pressure, heat, freezing, and storage.

Starch

It is recognized that the physical nature of starch is altered readily by the various treatments it receives. Starch absorbs water and when gelatinized, the starch granules become elongated and flexible (40, p. 47). Shrinkage of starch granules by dehydrating agents used in preparing specimens for microscopic study is not uncommon (40, p. 44). When placed on a slide with pressure applied by a cover glass for microscopic examination, the granules are distorted. They lose the birefringence which is typical of starch viewed by polarized light (27, p. 158). This birefringence is also lost as starch is cooked (9, p. 190).

For years, those interested in starch paste have been concerned about the effect of freezing upon gelatinized starch. As early as 1844, Scharling accidentally froze a paste and found that, when thawed, it was reduced to a

"soup" (48, p. 23).

Corn and wheat starch gels frozen at -2° to -3°C . (-28° to -27°F .) become very sponge-like and most of the water can be squeezed from them. A microscopic change is also noted. Very heavy veins appear which seem to correspond with the fibrous nature of the frozen gel (47, p. 234-235). This change is recognized as retrogradation, which refers to the tendency of linear starch molecules to attract and associate with one another (28, p. 43). Such a gel cannot be regelatinized to the original state (48, p. 41).

Rapid freezing results in much less retrogradation. Minus 25°C . (-13°F .) was found to be the critical temperature for corn and wheat starch gels. Above this, retrogradation was great but below this it was not (48, p. 41). Albrecht, Nelson, and Steinberg found that when frozen in liquid nitrogen simple starch gels retained their original properties. There were no changes after storage at -18°C . (0°F .) for one week. Ordinarily considerable retrogradation takes place during the first few days of such storage. They attribute this stability to the formation of smaller ice crystals which decreases the likelihood of molecular alignment necessary for retrogradation (1, p. 62). In continuing the study of freezing starch systems in liquid nitrogen, Albrecht and co-workers worked with white sauces

made of the same starches as were used in the starch-water studies. Samples were evaluated for smoothness, liquid separation, mouth feel, flavor, and general acceptability. In contrast to the freezing of the simple starch gels, they found that freezing white sauce in liquid nitrogen as compared with freezing at -18°C . (0°F .) did not result in significant differences. This difference in behavior between starch-water systems and white sauces is probably due to the addition of fat, milk, and salt to the starch-water system, and to an extremely complicated interaction between these factors (2, p. 66).

Protein

Protein is not unaffected by the various physical treatments. Slow freezing causes denaturation of the caseinate system of milk, but it is not noticed until stored several months at -18°C . (-0°F .) (46, p. 285). For minimum denaturation milk should be stored below -23°C . (-10°F .), but the temperature need not be less than -29°C . (-20°F .) (7, p. 5). Denaturation of the casein complex in frozen skimmilk can also be retarded by the addition of cane sugar (39, p. 752).

Foaming during dehydration causes changes in protein due to surface action (10, p. 15). Dehydrating agents themselves cause proteins to shrink and to lose their

native solubility (41, p. 292).

Fat

Fat is more susceptible to distortion than is either starch or protein. It is altered or completely dissolved by ordinary fixation methods (41, p. 292) used when preparing material for microscopic study.

Because of the structure of plastic solids such as fat, it behaves as a rigid solid when subjected to small stresses such as the force of gravity. Increased stress, however, causes it to flow like viscous liquids. Removal of the stress allows the plastic solid to regain the characteristics of a rigid solid (16, p. 195). The viscosity of fats and lipids in a substance is much more susceptible to changes of pressure than is that of protein or other constituents (42, p. 401). If cooky dough is mounted under a cover glass with water, fat is seen as globules, but if water is omitted, it may be seen in layers between or enveloping the starch granules. This artifact was found, by Platt and Fleming, to be due to the surface tension of water used in preparing the slides. The water also softened the more or less solid walls surrounding the fat (38, p. 391-392).

The physical state of fat is also dependent upon temperature. Hardness of butter, for example, is

empirically related to temperature (42, p. 402). Temperature also affects the mobility of plastic fats and oils (30, p. 536).

The oil-in-water emulsion of milk or cream is destroyed by slow freezing, so that fat separates from the hydrophilic phase. This is minimized by the addition of cane sugar or by rapid freezing (46, p. 285).

Alcoholic Dehydration of Specimens for Microscopic Study

The usual dehydrating solutions cause considerable tissue shrinkage and distortion. This is not critical when studying animal tissue as the shrinkage is uniform and distortion of the appearance is minimum. Animal tissue is therefore usually dehydrated in a graded series of ethyl alcohol. However, distortion is great in plant tissue because the cellulose walls do not contract as much as the cell contents (14, p. 98).

Probably, the most popular way of preparing botanical tissue for microscopic examination is the tertiary butyl alcohol method (14, p. 100). Johansen considers it the most satisfactory. Dehydration is begun in a solution of water, ethyl alcohol, and tertiary butyl alcohol, the total alcohol content being approximately 50 per cent. The concentration of alcohol is increased gradually to minimize

damage to the tissues. The final transfer is to 100 per cent tertiary butyl alcohol (23, p. 130). Tertiary butyl alcohol is considered an ideal dehydrating fluid because it mixes equally well with water, ethyl alcohol, balsam, and paraffin (23, p. 17).

Following dehydration, the tissues are cleared of the dehydrating medium if it is not miscible with paraffin. They are then infiltrated with and embedded in paraffin which serves to support the tissues when sectioned for the preparation of slides. The sections thus prepared are mounted on microscope slides. After removing the paraffin, they are ready for microscopic examination.

The Histological Freeze-drying Technique

Principle

The histological freeze-drying apparatus was developed so that tissues could be dehydrated without coming into contact with liquids which might disrupt the structure while dehydrating the tissue. The method was first introduced in 1890 by Altmann (19, p. 293). It received little recognition until 1932 when Gersh modified the technique. In 1933 Scott, and in 1936 Hoerr, published more basic work concerning the effect of the freeze-drying process upon the end product (43, p. 173). Newly designed

equipment is continually being developed in an effort to produce more representative samples in less time, but the basic principle remains the same (21, p. 572).

Freeze-drying is based upon the physical phenomenon of sublimation, whereby ice passes directly from the solid to the vapor state. The vapor pressure at the surface of the sample must be appreciably higher than the vapor pressure of the water produced by the condenser surface or other water removing means (11, p. 296). As the moisture is released by sublimation it is trapped by a desiccant or in the condenser trap of the freeze-drying apparatus (21, p. 572).

That freeze-drying produces a tissue nearly identical to its fresh counterpart, is based upon the assumption that it is frozen instantaneously. One of the primary factors involved in rapid freezing is the choice of freezing medium. Most workers tend to prefer that with the lowest melting point, feeling that it will provide the most rapid freezing. It is held by some, however, that rapid freezing is limited more by the heat conductivity of the already frozen surface of tissue than by that of the freezing medium (10, p. 22) (41, p. 292). It is their thinking, that it is wasted effort and expense to use a refrigerant with a lower temperature than that at which

heat is conducted by the tissue.

Following the theory that the most rapid freezing medium available assures the best results, liquid nitrogen with a melting point of -210°C . (-346°F .) (18, p. 419) is recommended by Hoerr (19, p. 296). However, it is often difficult to freeze tissue rapidly in liquid nitrogen (19, p. 295) because it boils at the low temperature of -196°C . (-321°F .) (18, p. 419). A protective envelope of gas forms around the comparatively warm tissue and retards the transfer of heat (19, p. 295). Freezing at -131°C . (-204°F .) in pentane, in a bath of liquid nitrogen, gives uniform fixation of tissue because this layer of insulation does not form. The most rapid freezing is obtained by using practical grade isopentane. This reagent also allows good transfer of heat, and its freezing point is below -160°C . (-256°F .) (19, p. 295-296). Perhaps one reason for more uniform fixation of tissue throughout the block when freezing in pentane or isopentane is that there is less contraction of the superficial layers of tissue after they have been frozen and before the interior of the block is frozen. It is probably also true that the freezing process is more rapid (19, p. 297).

The size of the blocks of tissue to be frozen should be related to the temperature of the freezing medium. Hoerr found that thick blocks of tissue frozen in liquid

air and dehydrated tended to show distortion toward the interior of the block. This was probably due to freezing of the surface and cooling of the exterior of the block to the temperature of liquid air before the interior was frozen. Such temperature differential causes great contraction-expansion change and thus mechanically places the interior of the block of tissue under great strain. Pure water is released most rapidly, thus causing a concentration of the salt content of the interior. This high concentration of salts causes the interior of the sample to freeze even more slowly (19, p. 296). When frozen directly in liquid nitrogen large pieces of tissue crack and break up. This cracking does not occur with very small pieces or when pentane is used for freezing (19, p. 299). Hoerr worked with pieces of tissue which were five millimeters in thickness. These were immersed in pentane at a temperature of -131°C . (-204°F .) and he seemed satisfied that they froze throughout in 15 seconds (19, p. 296).

The effectiveness of freezing and dehydrating the blocks of tissue is based not only upon the freezing medium, and the size of the block frozen, but upon the nature of the material as well. According to Scott and Hoerr, a loose and lobular arrangement of tissue allows the most satisfactory dehydration because it allows the

formation, in the connective tissue, of ice crystals from the surface inward. The ice thus formed conducts heat better than liquid water. Tissue which has a low fat and low water concentration is the most successfully frozen (41, p. 292). High water content may cause ice crystal artifacts (43, p. 179).

The maintenance of low temperatures throughout the freeze-drying process has been repeatedly emphasized. The rapidly cooled tissue is placed in a high vacuum system which has a temperature of -30°C . (-22°F .), or lower (44, p. 106). Rapid evaporation of cold isopentane adhering to the tissues at the start of evacuation of the freeze-drying chamber prevents undue temperature rise before equilibrium conditions are established (41, p. 295). Dehydration must be carried out below the eutectic point to prevent ion diffusion. Certain salt systems have a eutectic point below -20°C . (-4°F .) (13, p. 4). Simpson (43, p. 180) suggests a dehydration temperature below -40°C . (-40°F .) for more uniform preservation than is possible at -30°C . (-22°F .). There appeared to be no added benefit in dehydrating at -50°C . (-58°F .).

The rate of drying is greatly decreased when the temperature is reduced below -33°C . (-27°F .) because there is a great reduction in vapor pressure of the free ice surface between -20° and -60°C . (-4° and -76°F .). There

are, however, advantages of this slower rate of evaporation: there is less chance of the tissue being torn apart due to internal strain; the theoretical eutectic point of the tissue is approached (19, p. 300).

Dehydration time varies with the material being treated. Animal tissue can be successfully dehydrated in four to twenty-four hours. Plant tissue is harder to dehydrate, taking two to six days (21, p. 572). According to Jensen there are several reasons that dehydration of plant tissue requires a considerably longer time. "The extremely high water content of the cells, the high solute concentration in the central vacuoles, the massive cell walls, the heterogeneous composition of the tissues and the areas susceptible to tearing under stress such as the cambium and endodermis" are all interfering factors (22, p. 36).

Dried tissue cooled to -30°C . (-22°F .) will rapidly condense and absorb water from the atmosphere. It must either be allowed to warm up to room temperature in the original dehydration chamber in vacuo, or it must be removed rapidly from the chamber and allowed to warm up to room temperature in a desiccator (19, p. 302).

The dehydrated tissue is then infiltrated with paraffin; a process which may require from four to twenty-four hours (22, p. 37).

Advantages of the Freeze-drying Technique

The advantages of the freeze-drying technique are numerous. Probably the most important is that there are no chemical changes such as dissolving of salts and fats by aqueous and lipid solvents used in ordinary fixation, dehydration, and clearing methods (44, p. 105). The only physical change that takes place is a loss of water (41, p. 292). Fixation is extremely rapid, providing almost instantaneous cessation of metabolic activity. There is a minimum of shifting of diffusible constituents because fluid is not used and fixation is immediate (13, p. 3). In most cases the solute remains evenly dispersed as the frozen solvent sublimates. The remaining residue is porous and occupies essentially the same space as in the original material. There is no foaming because the material is frozen in the solid state and changes due to surface action do not occur (10, p. 15). There is a minimal tendency for coagulation of even lyophobic sols because the molecules of solute and colloiddally dispersed particles are "locked" in position as the solvent evaporates (10, p. 16). Absence of cell shrinkage is still another advantage of the freeze-dried tissue over the conventionally dehydrated one (13, p. 3).

Disadvantages of the Freeze-drying Technique

If tissue is improperly handled, large ice crystals form which disrupt the cell structure. However, if freezing is rapid enough, the ice crystals formed during the freezing of water are less than microscopic size and the constituents are not displaced to an extent that can be noticed microscopically (41, p. 292). Possibly greater damage due to slow freezing is denaturation of protein by dehydration resulting from the conversion of all free water to ice (34, p. 519).

Efficient drying at low temperature is not easily accomplished, but the dehydration must take place below the eutectic point of the tissue to prevent displacement of any constituent after it has been frozen (41, p. 292).

Fixation and Staining of Fat with Osmic Acid

As early as 1804, Tennant observed that organic materials blackened in the presence of osmium tetroxide, better known as osmic acid. He assumed that the blackening was caused by a deposition of metallic osmium (3, p. 458). Romeis felt that the osmic acid, a good oxidizing agent, was reduced to osmium dioxide and metallic osmium; the fat being oxidized at the double bonds and the heavy metal being adsorbed by the fat (26, p. 18). Gray (15, p. 525) says that the exact nature of the material laid

down in tissues when they are exposed to osmic acid is not known, but that it is fairly certain that it is not metallic osmium. Gatenby and Painter state that it is a hydrated form of one of the lower oxides (12, p. 29). Bahr states that some reaction products are lower oxides, but others are esters or complexes of the tetroxide (3, p. 469). Regardless of the form in which it is laid down, osmic acid is effective for immobilizing the fat (15, p. 52) and for clear-cut staining (15, p. 525). According to Romeis, the heavy osmium is adsorbed by the fat and thus protects the fat from being dissolved by its usual solvents (24, p. 30).

Osmic acid will become reduced on the surface of many organic materials but the oxides are laid down first on the fatty constituents and, later, on other components of the cell and cell wall. It will react only with unsaturated fats (3, p. 471). The fatty acid most commonly acted upon is oleic acid (49, p. 41). Carbohydrates and nucleic acids are inert toward osmic acid (3, p. 471). Some of the amino acids, examples being tryptophan and cysteine, reduce it very rapidly; others more slowly (37, p. 90). The total amount of these two amino acids would not exceed 0.2 per cent of the weight of the cake batter used in this study. This figure was calculated from data presented by Leverton (29, p. 71-73).

When used for killing and fixing, osmic acid is most commonly made into solution, the concentration of which should be less than two per cent (23, p. 32). However, Johansen states that the osmic acid fumes or vapor are as effective as the solution (23, p. 37), and Gray agrees that the vapor phase may be employed for fixation (15, p. 193). Fixation is complete as soon as the material has become brown throughout. Under ordinary circumstances killing fluids containing osmic acid should be washed out before the sections become black, or they will eventually become a charred mass (23, p. 32).

Taking advantage of the blackening which accompanies the oxidation of organic materials by osmic acid, several workers have used it to simultaneously stain and fix the fat for microscopic examination. Burhans and Clapp do not elaborate upon the method they used to apply osmic acid vapors to their samples of bread dough. Following the example of Lowe and Nelson (31, p. 164) and Morr (35, p. 28), who worked with baked cake, Jooste and Mackey (26, p. 18) prepared a five per cent aqueous solution of osmic acid. This they placed in a five milliliter beaker, surrounded by a nest of glass wool. Small samples of baked cake were placed on the glass wool and the whole was covered with a small bell jar. The edges were sealed with stop-cock grease. This was kept in the dark and the osmic

acid penetrated nearly to the center of the blocks in seven hours. Following this treatment the samples were dehydrated in alcohols and embedded in tissuemat.

CHAPTER III

PROCEDURE

As orientation, a brief summary of the procedures used is given here. A description of the preliminary work as well as final procedures then follows.

Two different fats were used for the study. Differences in behavior between the two might be determined, and because they represent diverse groups of fats a wider range of information might be obtained. As it seemed quite certain that the microscopic images of the two fats would be different when incorporated in cake batter, their use should aid in the evaluation of the freeze-drying apparatus as a tool for study of batters and doughs.

Two replications of batters made with each fat were prepared. They were quick-frozen, dehydrated, exposed to osmic acid vapors to fix the fat, embedded in paraffin and sectioned on a rotary microtome. The finished slides were examined to determine the dispersion of air cells and the location of fat with reference to other constituents of the batter.

Certain organoleptic properties were determined for cakes baked from each type of batter.

Ingredients

Two different fats were used for this work. One was a mixed triglyceride (Melvo) and the other a mixed triglyceride plus an emulsifying agent (Crisco). To avoid confusion they shall hereafter be referred to by their trade names.

Sufficient quantities of all staple ingredients were purchased to last throughout the study. Included were: Crisco, Melvo, dried nonfat milk (Starlac), cake flour (Swans Down), granulated sugar, tartrate baking powder (Royal), salt, and vanilla extract. Fresh eggs were purchased as needed.

Approximately one-third of the water used to prepare the batter for microscopic study was a three per cent solution of eosin Y, a water soluble stain.¹ It stained the starch and protein pink, but after subsequent treatments the protein appeared tan and the starch unstained. The fat was stained black by the action of osmic acid vapors.²

1. Eosin Y: Yonkers Laboratory Supply Company, 70 Palisade Avenue, Yonkers 2, New York

2. Osmium tetroxide: Merck and Company, Incorporated Chemical Division, Rahway, New Jersey

Formula and Method of Mixing

The formula and method of mixing were developed for laboratory studies of fat behavior in cake. They are given in Tables 1 and 2.

Table 1
Cake batter formula

<u>Ingredients</u>	<u>Weight</u> grams
Fat, hydrogenated vegetable	45.0
Sugar, granulated	120.0
Flour, cake	100.0
Milk, dried non-fat	13.0
Salt	0.7
Baking powder, tartrate	3.75
Egg yolk	12.0
Egg white	20.0
Water, distilled	80.0
Vanilla, 1 teaspoon	--.-

Preliminary Directions:

1. Divide water into three portions. Save 25 ml. to be added to the creamed fat mixture. For microscopic study substitute 25 ml. of three per cent solution of eosin. Divide remaining water into two 27.5 ml. portions to be added to egg yolk.
2. Divide egg yolk into two 6 gm. aliquots. To each add 27.5 ml. water. Stir occasionally to keep egg yolk suspended in water.
3. Divide flour, dry milk, and salt each into two equal portions (50.0 gm., 7.5 gm., and 0.35 gm. respectively). Sift the two portions of dry ingredients separately.
4. Save the baking powder to be added near the end of mixing.

Table 2
Method of mixing

<u>Steps</u>	<u>Ingredients</u>	<u>Mixing Speed</u>	<u>Time</u>
1	Cream fat, sugar, and vanilla. Scrape bowl after each two minutes.	8 (med. fast)	8 min.
2	Add 25 ml. water	3 (slow)	1 min.
3	Scrape bowl. Add $\frac{1}{8}$ dry and $\frac{1}{8}$ liquid ingredients. Scrape bowl after each 25 seconds.	3 (slow)	50 sec.
4	Add remaining dry and liquid ingredients.	3 (slow)	25 sec.
5	Scrape bowl. Add baking powder.	3 (slow)	25 sec.
6	Scrape bowl. Beat egg white stiff and add. Scrape bowl after each 15 seconds.	3 (slow)	1 min.

Batters were mixed with a Sunbeam Mixmaster, Model 10A1.

The fat control was prepared by whipping 175 grams of fat at speed 8 of the same mixer for five minutes.

Baked cakes for organoleptic study were prepared as above. 120 grams of batter were baked in pans of eight ounce capacity for 19 minutes at a temperature of 190°C. (375°F.).

Preliminary Investigation

Much of the preliminary work was done with fat only, usually Crisco, into which air had been whipped. This presented a sample which was relatively simple in structure and composition. The effects of manipulation were much more readily observed than would have been the case had the fat been in a complex cake batter. The problem of differentiating the direct effects of the manipulation on fat from the indirect effects of changes in other constituents was also avoided.

Obtaining Samples for Dehydration

To obtain a sample of cake batter for dehydration presented a particular problem. Its fluidity prevented a simple sectioning of a suitably thin sample. It was necessary to treat the batter so that it became a solid, and thereafter could be handled as such. The primary objective was that the physical structure be preserved as nearly as possible.

Several methods of obtaining the samples for dehydration were attempted:

Batter introduced into one-fourth inch molds. Batter was introduced into an aluminum foil mold one-fourth inch in diameter. This was placed on dry ice until the batter

was frozen, a process which took at least two minutes. When solid, the foil was peeled off and the sample was considered ready for the dehydration process.

Batter frozen on bed of crushed dry ice. One to two milliliters were poured onto a bed of crushed dry ice. In about three minutes the block was frozen solid and at this time samples one to three millimeters in diameter could be chipped off with a chilled scalpel.

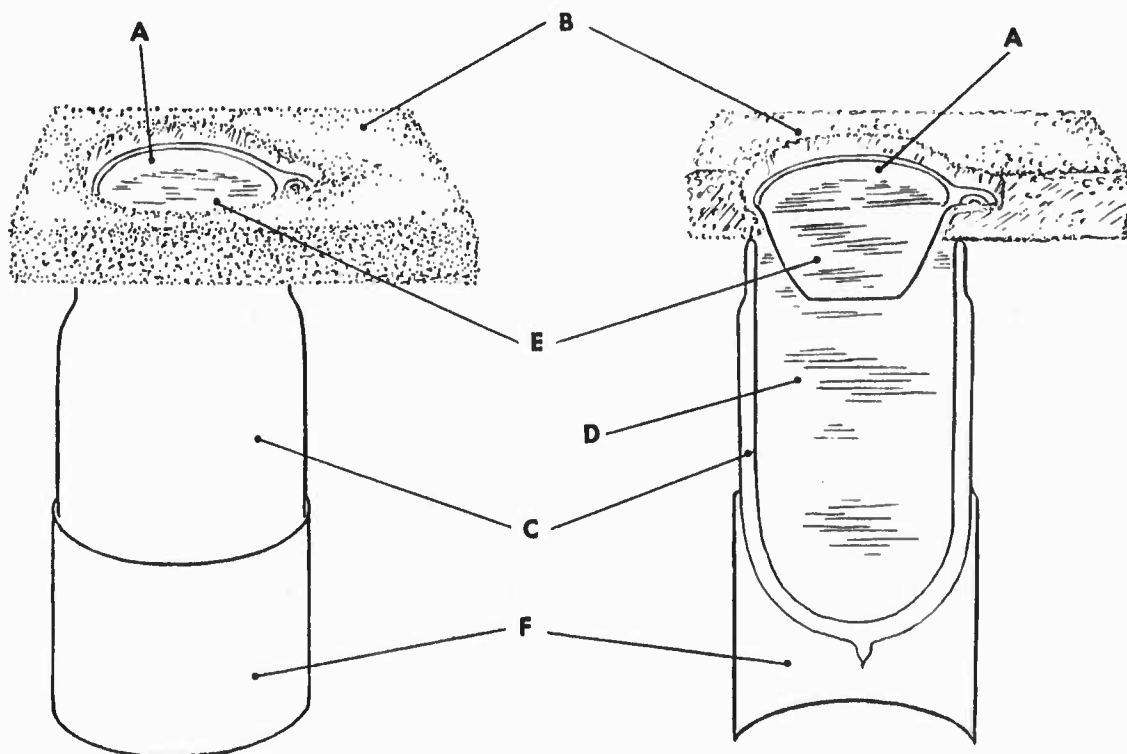
Batter dropped into chilled isopentane. Batter was dropped into isopentane chilled in a large test tube to a temperature near its freezing point, -160°C . (-256°F .) in liquid nitrogen.³ The batter appeared to freeze instantaneously.

Batter frozen in isopentane chamber. A chamber (Fig. 1) was erected which facilitated handling of the sample in the isopentane. Isopentane was placed in a small aluminum cup, shallow enough that the bottom could be used as a cutting surface. This cup rested on a styro-foam collar, allowing the cup to be partially immersed in liquid nitrogen. When the isopentane became very viscous it was considered to be near its freezing point. It was allowed to warm very slightly and a sample of about

3. 2-Methylbutane, Practical grade, Eastman Kodak, Rochester 3, N.Y.

Figure 1

Equipment used for rapid freezing of samples of fat and cake batter in isopentane chilled with liquid nitrogen.



- A - Aluminum cup
- B - Styrofoam collar
- C - Dewar flask
- D - Liquid nitrogen
- E - Isopentane
- F - Supporting sleeve

1 milliliter of batter or fat was immersed in it, using a small spatula as the transferring implement. This sample appeared to freeze instantaneously. It was impossible to time the freezing process with a laboratory stopwatch. While held in the isopentane in liquid nitrogen to prevent thawing, samples as small as one millimeter in diameter were obtained by chipping the frozen block with a scalpel.

Samples were transferred directly to coded cassettes which were on dry ice or in chilled isopentane, depending upon the freezing medium used, until all were ready for the dehydration process. Of particular note concerning samples to be freeze-dried, the cold isopentane adhered to tissues at the start of evacuation in the freeze-drying chamber. This prevented a rise of temperature before equilibrium conditions were established.

Dehydration by Freeze-drying

Freeze-drying was carried out in a histological freeze-drying apparatus.⁴ This consisted of a horizontal glass manifold with ground glass connections to two lyophil tubes and a condenser trap. Each tube contained a perforated metal cassette assembly which held and identified up to ten tissue samples. A glass boat containing a

4. Model FD-11, Scientific Specialties Corporation, Cambridge, Mass.

chemical desiccant was inserted through a ground glass connection near the condensor trap. A high vacuum pump was connected to the system to draw the moisture to the trap.

The freeze-drying apparatus was prepared for the process immediately before the batter was mixed or the fat whipped. A fresh supply of desiccant was placed in the glass boat to pick up any traces of moisture. Dewar flasks were placed around the corked lyophil tubes and the condensor trap. The dewar flasks were packed with dry ice. Acetone was added to the dry ice cooling the condensor trap to increase the efficiency. This prevented moisture from being drawn into the vacuum pump. All ground glass joints were lubricated with a high vacuum lubricant. The pump was connected to the apparatus and the system evacuated.

When all samples were prepared, the cold cassettes in which they were placed for segregation and identification were placed in cassette holders which were in turn lowered into the cold lyophil tubes. These were corked temporarily to minimize moisture condensation. As rapidly as possible they were attached to the manifold. The pump was turned on and the system was evacuated. Within an hour the pressure was well established between 0.01 and 0.001

millimeters mercury. The dehydration process was timed from this point.

When a new type of tissue is dehydrated by freeze-drying it is necessary to determine the approximate time necessary for the process. As mentioned previously, recommended temperatures during dehydration are usually between -30° and -40°C . (-22° and -40°F .). These temperatures were difficult to maintain throughout the five days required for dehydrating the tissue. Special refrigeration equipment would have been needed. A choice was necessary between allowing the samples to warm to room temperature after several hours of dehydration as was recommended by the manufacturer of this particular apparatus, or holding them at dry ice temperature, -79°C . (-110°F .), which would increase drying time markedly.

In order to determine the time required for dehydration, a batter was prepared, and from this, samples as nearly identical as could be obtained were treated in five ways. Early work indicated that at least three days would be required to dehydrate the batter. Possibly this time would be sufficient for those samples allowed to warm to room temperature while dehydrating. Whipped fat controls were dehydrated for 120 hours, one at room temperature and the other at dry ice temperature.

Cake batter samples dehydrated at room temperature.

Three groups of samples (12 in all) were quick-frozen before they were placed in the lyophil tubes. Dry ice was packed around the tubes and allowed to evaporate without replacement. Thus for most of the dehydration period, the samples were at room temperature. They were held in the apparatus for 72, 96, and 120 hours. Dry ice was replaced around the condensor trap every 20 hours for the entire dehydration time.

Cake batter samples dehydrated at dry ice temperature.

Two groups of samples (eight in all) were held at dry ice temperature throughout the dehydration period. These were dehydrated for 96 and 120 hours. Dry ice was replaced around both the lyophil tubes and the condensor trap every 20 hours.

Dehydration with Alcohol

The purpose of the major part of the work reported in this thesis was to develop a method for the use of the freeze-drying apparatus for the study of cake batters; however, in view of the fact that dehydration with alcohol is a time-honored method, a comparison of the two methods was deemed desirable.

At room temperature and employing alcoholic solutions of concentrations normally used to dehydrate tissue, the batter disintegrated before dehydration took place. Because of the necessity of handling the batter as a solid, lower temperatures were tried. At -20°C . (-40°F .) the batter was firm, but rather sticky. Apparently some constituents prevented freezing. A temperature approaching that of dry ice was used, at which the batter appeared to be a solid. However, tertiary butyl alcohol and the lower concentrations of ethyl alcohol in which dehydration is usually begun, solidify at this temperature. Therefore, dehydration was begun in 95 per cent alcohol.

Vials containing 95 per cent alcohol were placed in a bath of alcohol and dry ice. After one hour, samples of fat and batter were dropped into this chilled mixture. The alcohol was changed three times; following intervals of 2, 36, and 2 hours, after which the batter appeared to be dehydrated.

Fixation of Fat with Osmic Acid

Samples placed under bell jar with alcoholic solution of osmic acid. The first method attempted to introduce osmic acid vapors into the samples of fat and cake batter after dehydration was based on that used by Jooste and Mackey (26, p. 18) for baked cake. Samples were placed on

a nest of glass wool surrounding a five milliliter beaker containing a one per cent alcoholic solution of osmic acid. The whole was covered by a bell jar four and one-half inches in diameter and allowed to stand in the dark at room temperature for 20 or 48 hours.

Samples placed in vacuum desiccator with alcoholic solution of osmic acid. A slightly modified approach was also tried. A one per cent alcoholic solution of osmic acid was placed in a small beaker in the bottom of a vacuum desiccator. The samples were suspended in a small nest of glass wool from a hook in the lid of the desiccator. The chamber was evacuated and the vacuum disconnected. The desiccator was placed in the dark at room temperature for 48 hours.

Samples placed directly in alcoholic solution of osmic acid. Samples were placed directly into a one per cent alcoholic solution of osmic acid for two and seven hours at atmospheric pressure and for two hours under a vacuum of 15 inches.

Samples placed in cold solutions of osmic acid and carbon tetrachloride. As osmic acid is very soluble in carbon tetrachloride (18, p. 418), an attempt was made to use this as the solvent. Although fats are dissolved in

carbon tetrachloride at room temperature, at lower temperatures they are less soluble. According to Bailey (4, p. 70), fats become less soluble in organic solvents below their melting points and at temperatures approaching that of dry ice all fats are but slightly soluble. Carbon tetrachloride freezes at -23°C . (-9°F .) (18, p. 695), making it impossible to immerse the fat in it at dry ice temperature, -79°C . (-110°F .). Osmic acid was dissolved in carbon tetrachloride to make a one per cent solution. The solution was frozen in small vials, the fat samples were placed on top of the solidified solution of osmic acid in carbon tetrachloride, and the vials were capped. The vials were held on dry ice for one hour to assure equalization of temperature. They were then placed in the freezer to gradually warm until the solution melted. As the samples dropped into the cold solvent, the vials were again placed on dry ice.

Evidence has been presented to the effect that it is not necessary to introduce osmic acid as a solution. Gray (15, p. 193) states that osmic acid "may be employed for fixation in the vapor phase". Johansen says that "the fumes or vapors kill just as efficiently as does the solution". Therefore, an attempt was made to introduce vapors from the solid osmic acid crystals.

Samples subjected to vapor arising from sublimation of osmic acid crystals. As crystals of osmic acid were known to sublime, an attempt was made to fix the fat with undiluted osmic acid. A desiccant and 0.5 gram of osmic acid crystals were placed in the bottom of a lyophil tube identical to that which held the samples during dehydration. The tube was placed in a dewar flask which was filled to about half the height of the lyophil tube with dry ice. The tube was corked and the entire assembly was allowed to remain at room temperature for 18 hours. As it warmed, the osmic acid sublimed and the vapors penetrated the sample.

To avoid possible changes of the samples which might occur if warmed to room temperature before osmic acid penetration was complete, this penetration was carried out at 6°C. (43°F.). At this temperature the structure of the fat would be more stable. The samples were removed after 48, 72, and 96 hours.

Preparation of Dehydrated Tissue for Microscopic Study

Using the methods considered best following preliminary investigation, permanent slides of cake batter were prepared. These methods are briefly stated below.

The batters were prepared as outlined in Tables 1 and 2. Using the chamber illustrated in Figure 1, isopentane

was chilled to near -160°C . (-256°F .) in liquid nitrogen. Approximately one milliliter portions of batter transferred to the isopentane froze instantaneously. While held in the isopentane to prevent thawing, samples one to two millimeters in diameter were chipped from the frozen block.

The samples were placed directly into coded cassettes which were immersed in chilled isopentane. When all samples were prepared, the cassette assemblies were transferred to chilled lyophil tubes. These were attached to the freeze-drying apparatus which was ready for operation. The vacuum pump was turned on and the system was evacuated. The dehydration process was timed from one hour after the pump was turned on, as this much time was allowed for the pressure to become well established between 0.01 and 0.001 millimeters mercury. The samples were dehydrated for 120 hours while the dry ice level was maintained in the dewar flasks around the lyophil tubes and the condensor trap to prevent temperature fluctuations.

When dehydration was complete, the cassette assemblies were transferred to an identical lyophil tube in the bottom of which was a desiccant and 0.5 gram of osmic acid crystals. The tube was corked and placed in a refrigerator at 6°C . (43°F .) for 114 hours. This was a longer time than had been found necessary to fix the fat samples. The

additional time was considered essential for the osmic acid vapors to penetrate to the fat dispersed in the center of the samples of cake batter.

The lyophil tube containing the cake batter or fat samples impregnated with osmic acid was removed from the refrigerator and allowed to warm gradually for one hour. The samples were then placed in vials of melted paraffin and held in a vacuum oven at 65°C. (149°F.) with 15 inches of vacuum for two hours.⁵ The paraffin was then changed and the vials were transferred to a paraffin oven for three hours. The paraffin was changed once more and after 16 additional hours in the paraffin oven the samples were embedded in blocks of paraffin.⁶

When cooled, the embedded samples were trimmed and mounted on wooden blocks which served to hold the samples in the microtome. To facilitate sectioning, the embedded samples were soaked in a five per cent solution of glycerine in 70 per cent alcohol for three to seven days (70, p. 140). The samples were sectioned to thicknesses of 15 and 30 microns on a rotary microtome. The sections were mounted on slides with Haupt's adhesive minus the formalin floating medium, as freeze-dried tissue should not be floated.

5. Fisher's tissuemat, melting range 60-63°C.

6. For detailed information on microtechnique, refer to Gray (14) and Johansen (23).

Paraffin was removed from the sections by immersion in xylol for five minutes and cover glasses were mounted with Canada balsam. To determine whether distortion was caused by xylol treatment photomicrographs were taken before and after removal of the paraffin.

The primary objectives in examining the slides were to determine the appearance and location of fat and the dispersion of gas cells in cake batter.

Organoleptic Characteristics

Two batters which were identical with the exception that one contained Crisco and the other Melvo, were prepared for organoleptic comparison. They were tasted by two persons and the following characteristics were compared: size of cells, thickness of cell walls, compactness, tenderness, fragility, springiness, volume, moistness and velvetiness.

CHAPTER IV
RESULTS AND DISCUSSION

Preliminary Investigation

Obtaining Samples for Dehydration

The following observations and decisions were made concerning the various methods attempted to obtain representative samples for dehydration.

Batter introduced into one-fourth inch molds. This method was considered unsatisfactory for several reasons. The structure of the batter might be distorted by forcing it into a mold. The sample was too large to freeze rapidly on dry ice. It was not cold enough when placed in the freeze-drying apparatus. Partial thawing or incomplete freezing was evidenced by the appearance of the sample when removed from the freeze-drying apparatus. Rather than having sharp edges as when unmolded, the dehydrated sample appeared puffy.

Batter frozen on bed of crushed dry ice. This method was also considered unsatisfactory. Although pains were taken to drop small bits of batter onto the crushed dry ice, such drops were apparently too large to be rapidly frozen by a medium of this temperature. As noted under Procedure, about three minutes were required. The contact

of the sample with the refrigerant appeared to be inadequate as relatively warm air almost surrounded the sample and freezing progressed from only one direction. Chips partially thawed as evidenced by the puffiness mentioned above. Another indication of slight thawing was that the dehydrated samples stuck to the cassettes. Such thawing probably indicates that the temperature of the samples was not sufficiently low when placed in the cassettes.

Batter dropped into chilled isopentane. It was impossible to drop samples small enough to be dehydrated as such. Because of this, it was necessary to remove the frozen batter from the test tube to be broken into smaller sections. During this process, the samples thawed considerably. Even if smaller drops could have been frozen, artifacts might have occurred prior to freezing as a result of surface tension forces of the small drop. This method required modification to achieve satisfactory results.

Batter frozen in isopentane chamber. Small cake batter samples dropped into isopentane at a temperature of -160°C . (-256°F .) appeared to freeze instantaneously. Hoerr seemed satisfied that tissue which was five millimeters thick froze in 15 seconds (19, p. 296). Even though the drops of cake batter were considerably larger,

they froze practically instantaneously. As explained earlier, chips from the frozen block of batter were prepared without removing it from the isopentane. Thus, bits small enough for efficient dehydration were obtained without thawing. They had the same sharp edges when dehydrated as when introduced into the cassettes and they did not stick to the cassettes. The structure did not appear to be crushed by the chipping process or in any way disturbed. This method was, therefore, considered best for preparing cake batter samples for dehydration.

Dehydration by Freeze-drying

Whipped fat samples. Microscopic examination of sections of the whipped fat controls led to the realization that the samples must be held at dry ice temperature during dehydration. After fixation with osmic acid vapors the sample which was treated as such maintained its aerated structure as evidenced by its lacy appearance (Fig. 2). Samples which were allowed to warm to room temperature during dehydration were no longer aerated. The vacuum had apparently caused the fat, which is normally solid within this temperature range, to become mobile. The air cells probably coalesced and were lost. The fat expanded, thus presenting a solid but cracked appearance (Fig. 3). It seemed safe to assume that air cells

Figure 2

Photomicrograph (100x) of whipped fat (Melvo) processed at dry ice temperature in the freeze-drying apparatus. 15 microns. The fat, stained black with osmic acid vapors, appears as a lacy network in which the air is incorporated.

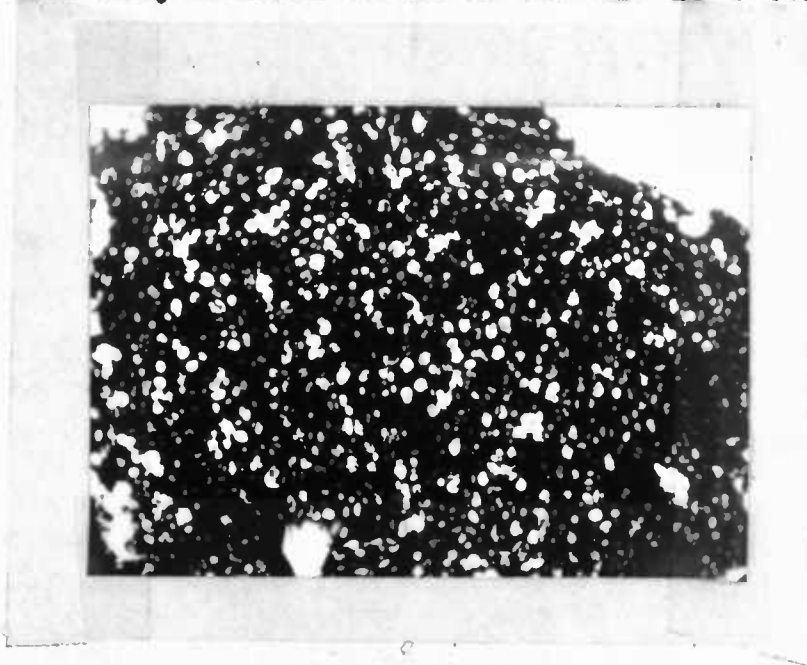


Figure 3

Photomicrograph (100x) of whipped fat (Melvo) processed at room temperature in the freeze-drying apparatus. 15 microns. The fat, stained black with osmic acid vapors, appears as a cracked, but unaerated solid.



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dispersed in fat in batter treated the same way would also coalesce.

The cake batter samples were carefully inspected when dehydration was completed. The following observations were made:

Cake batter samples dehydrated at room temperature.

All samples held at room temperature were well dehydrated, but this method was considered unsatisfactory because of possible changes in the fat location or aeration. These possibilities were substantiated by reference to the whipped fat samples described above.

Cake batter samples dehydrated at dry ice temperature.

Samples dehydrated for 96 hours at dry ice temperature were a little soft upon removal from the freeze-drying apparatus. They melted slightly when handled. Samples dehydrated for 120 hours at dry ice temperature were, however, dry, very hard, and seemed to possess the same sharp edges as when the dehydration was begun. As microscopic sections of whipped fat treated in this manner appeared to be non-distorted, the method of dehydrating cake batter at dry ice temperature for 120 hours was considered the best.

Dehydration with Alcohol

Dehydration with alcohol at -72°C . (-98°F .) seemed to be complete, but no better than that accomplished by freeze-drying. Even at such a low temperature it was noticeable that the outer surfaces were washed away, indicating that this method of dehydration cannot produce a non-distorted sample of cake batter for microscopic examination.

Fixation of Fat with Osmic Acid

Samples placed under bell jar with alcoholic solution of osmic acid. Osmic acid impregnation of fat samples was incomplete. The interior was not fixed and it dissolved during infiltration of the specimen with paraffin. Only the peripheral area was fixed and stained black with osmic acid. The sections appeared as black lacy shells (Fig. 4). Impregnation was not noticeably improved by increasing the time of exposure to osmic acid vapors. The same observations were made on samples of dehydrated cake batter.

Samples placed in vacuum desiccator with alcoholic solution of osmic acid. Samples treated in this way were no more satisfactory than those held under the bell jar.

Figure 4

Photomicrograph (100x) of a segment of a whipped fat (Crisco) sample incompletely fixed with osmic acid vapors. 15 microns. Only the peripheral area is penetrated, allowing dissolution of the interior upon infiltration with paraffin.



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City of BROWN Paper

Samples placed directly in alcoholic solution of osmic acid. Samples of cake batter immersed in the solution were completely penetrated with osmic acid, but this method was not considered satisfactory because samples softened and were partially washed away.

Samples placed in cold solution of osmic acid and carbon tetrachloride. This method, used with fat samples only, was unsatisfactory. The fat samples dissolved before they reached a temperature low enough that they would be insoluble in carbon tetrachloride or were fixed by the action of osmic acid.

Samples subjected to vapor arising from sublimation of osmic acid crystals. This method allowed complete penetration of osmic acid into the fat samples. It was, therefore, considered a satisfactory method to be used throughout the study. There are several advantages associated with the method of using solid crystals of osmic acid in the lyophil tube equipped to serve as a desiccator. A minimum of handling provides the least opportunity for the dehydrated sample to pick up moisture. Temperature is more easily controlled and the warming process is more gradual. There is less danger of misidentification as the samples are not removed from the coded cassettes in which

they were dehydrated until infiltration with paraffin is begun. Minimum handling of the highly corrosive osmic acid provides a safety factor and is also an added convenience. The expense is minimized as 0.5 gram is sufficient for two or more replications. When dissolved in alcohol, the osmic acid must be used immediately and the waste is great.

Penetration was satisfactory when carried out at 60°C. (43°F.) for 48 to 96 hours. This temperature was, therefore, used when introducing osmic acid into the tissue.

Microscopic Study of Dehydrated Tissue

Sectioning Qualities

Samples were sectioned to thicknesses of 15 and 30 microns. The aeration of the whipped fat samples was clearly noted at 20 microns or less. At 10 microns the samples were compressed, regardless of precautions taken in handling the material. Therefore, although the image of samples cut to 10 microns or less might have been clearer when viewed under high power (8, p. 97), a thickness of 15 microns was accepted as best for viewing sections of whipped fat and for studying the dispersal of fat in cake batter. A thickness of 30 microns seemed

necessary for studying the hydrophilic constituents in the cake batter because of the fragility of the structure and because the stain was not sufficiently intense in thinner sections. Both thicknesses were valuable in studying the relation of the various constituents to each other.

The samples of whipped Melvo seemed to section more easily than did the samples of Crisco. The air spaces in the whipped Crisco were apparently larger, allowing larger masses of paraffin to form. These paraffin clumps interfered with sectioning by tearing away from the surfaces of the fat particles.

Penetration of Osmic Acid Vapors

In all cases when treated as described on page 42, the fat samples were completely penetrated by the osmic acid vapors. The black lacy network, found to be typical of whipped fat, extended throughout the sections.

Penetration of the fat particles with osmic acid was almost always complete in samples of batter prepared with Melvo. However, the center of the samples prepared with Crisco was usually devoid of fixed fat (Fig. 5). Occasionally, stained fat was noted in the extreme center (Fig. 6). In the intermediate zone, there appeared to be no fat while the exterior zone of the sample was well stained in reference to the fat. The cause of this

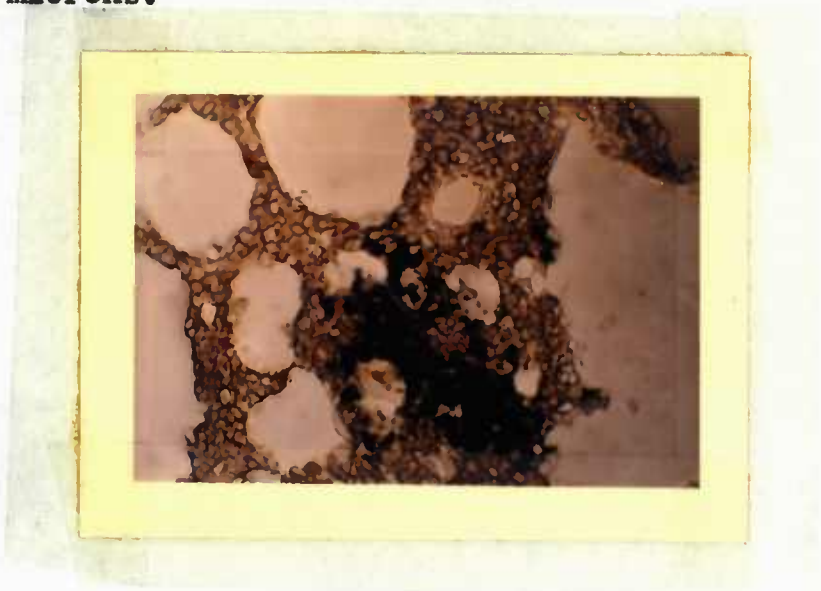
Figure 5

Photomicrograph (67x) of cake batter prepared with Crisco, apparently incompletely penetrated with osmic acid vapors. 15 microns.



Figure 6

Photomicrograph (67x) of fat fixed with osmic acid within an intermediate unfixed zone, appearing in cake batter prepared with Crisco. 30 microns.



incomplete penetration is not known. It appears, however, to be due to some treatment following the freezing of the batter, as the final sample, in which this border of fixed fat was noted (Fig. 5) was only a small segment of that initially frozen. It may be directly or indirectly associated with the nature of the fat, as this irregularity was not noted in batters prepared with Melvo and treated simultaneously.

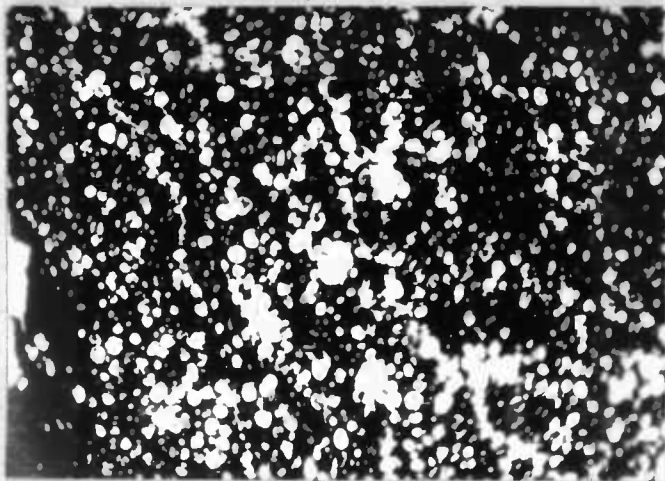
By earlier experimentation, it was determined that hydrophilic constituents of this batter did not become black as did the fat, even on direct contact with osmic acid. Some of them assumed a brown tinge. Likewise, in the sections treated as described on page 43, only the fat stained black, even at the extreme surface of the sample.

Structure of Whipped Fat

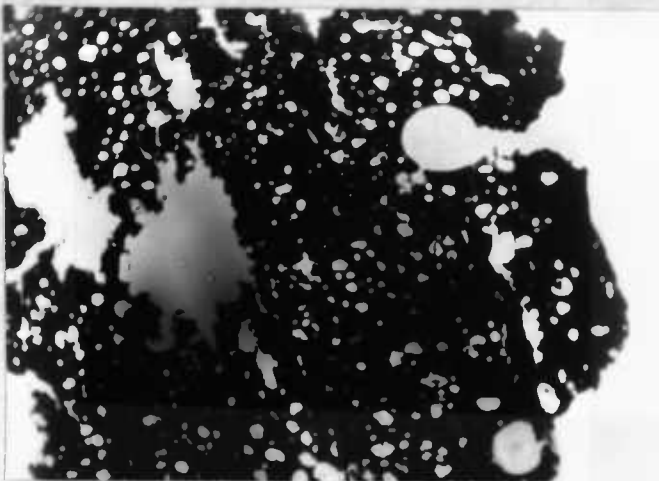
When whipped, samples of Melvo incorporated more air in smaller cells which were more highly dispersed than samples of Crisco. The thinner intercellular spaces and slightly smaller cells may be seen at various magnifications in Figures 7, 8, and 9. This finer dispersion of air in whipped Melvo was associated with the ease with which the Melvo samples sectioned, as was speculated on page 55.

Figure 7

Photomicrographs (100x) of whipped fats after having been processed in the freeze-drying apparatus and fixed with osmic acid vapors. 15 microns.



Melvo



Crisco



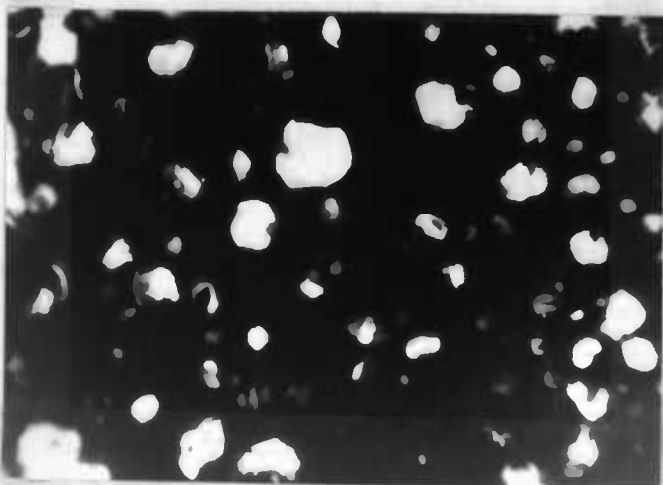
Chillbrow Paper

Figure 8

Photomicrographs (450x) of whipped fats after having been processed in the freeze-drying apparatus and fixed with osmic acid vapors. 15 microns.



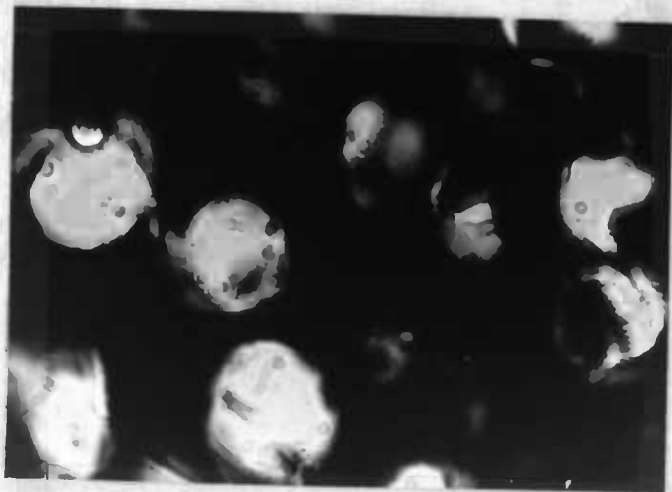
Melvo



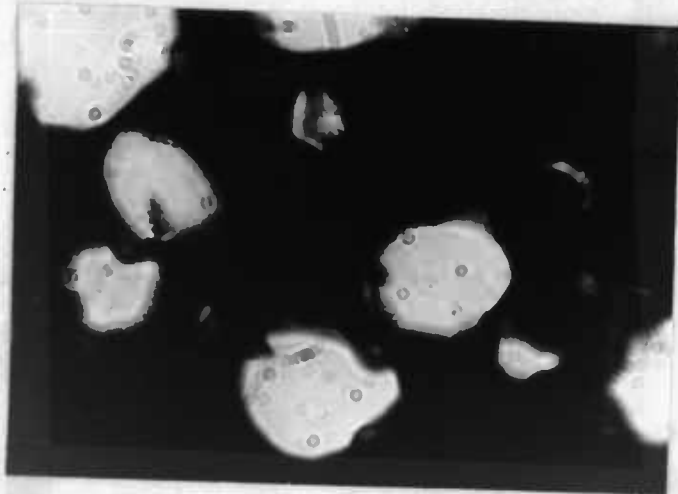
Crisco

Figure 9

Photomicrographs (1000x) of whipped fats after having been processed in the freeze-drying apparatus and fixed with osmic acid vapors. 15 microns.



Melvo



Crisco

Cake Batter Structure

In both types of cake batter the air appeared to be emulsified in a matrix of hydrophilic constituents (Figs. 10 and 12). Incorporated in this matrix were starch granules and fat particles. Evidence that the cell walls were probably formed by the hydrophilic constituents is the continuity of the phase stained by the water-soluble dye.

Cake batter prepared with Melvo. Melvo appeared in the batter as small angular fragments. Frequently the starch granules were partially or entirely incased by the fat (Fig. 10). Fat occasionally appeared as part of the air cell walls, but it was no doubt lined with the hydrophilic phase (Fig. 11).

Cake batter prepared with Crisco. The particles of fat in the batter prepared with Crisco tended to have a globular form (Fig. 13). They appeared to be more highly dispersed than the particles in the batter prepared with Melvo. Although the fat globules were frequently clumped, they did not coalesce.

These observations of fat in cake batter differ from those of Carlin (9, p. 198), Mackey (32, p. 261), and others in the field. They report that when batters are compressed under a coverglass for microscopic examination

Figure 10

Photomicrograph (67x) of freeze-dried cake batter prepared with Melvo. 15 microns. Tan hydrophilic constituents form the continuous phase of the aerated batter in which may be seen unstained starch granules and angular fragments of fat stained black with osmic acid vapors. Starch granules also appear in the fat.



Figure 11

Photomicrograph (300x) of freeze-dried cake batter prepared with Melvo. 15 microns. The angular fat particles are present around the periphery of air cells within hydrophilic constituents as seen in upper right and lower right corners of photomicrograph. Unstained starch granules may be seen in the fat and in the hydrophilic phase.



Figure 12

Photomicrograph (67x) of freeze-dried cake batter prepared with Crisco. 30 microns. Primary structure is an aerated matrix of hydrophilic constituents in which fat particles and starch granules are dispersed.

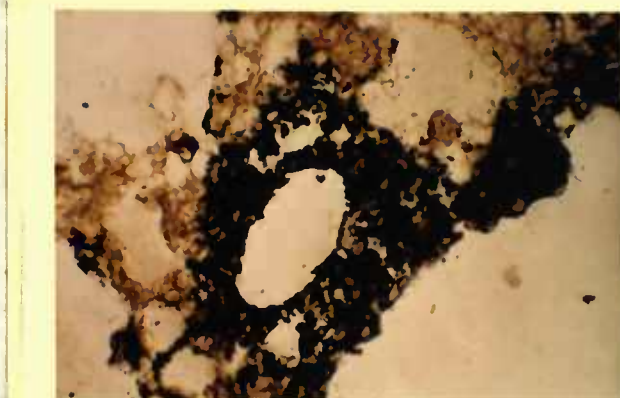
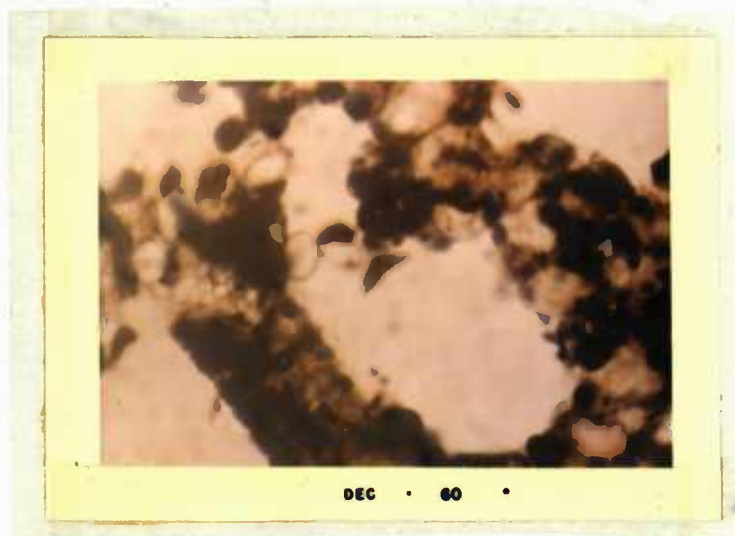


Figure 13

Photomicrograph (300x) of freeze-dried cake batter prepared with Crisco. 15 microns. Globular fat particles stained black with osmic acid and unstained starch granules are incorporated in a matrix of tan hydrophilic constituents.



the air appears to be held in the fat.

The presence of an emulsifier appeared to affect the aeration of the fat as well as the distribution of the fat in the cake batter. In the whipped fat less aeration was noted when the emulsifier was present. In emulsifier-containing batters, less aeration was observed by Jooste and Mackey (25, p. 190). In freeze-dried specimens Crisco particles assumed a more globular form than the Melvo which contained no emulsifier.

Organoleptic Characteristics

The following qualities were noted when a cake prepared with Crisco was compared with one prepared with Melvo. The batter containing Crisco produced a cake with smaller cells with slightly thinner cell walls, although the cells in this cake had more variation in size. The cake prepared with Crisco was lighter, more tender, more fragile, slightly springier and had larger volume. It seemed more moist and more velvety than the cake containing Melvo.

Using the formula given on page 30, Jooste and Mackey obtained essentially the same results (25, p. 194). By specific gravity determination, they found that the batters prepared with additional emulsifier were less aerated than those without added emulsifier yet produced

the larger cakes (25, p. 190). As was pointed out previously, whipped Crisco was less aerated than whipped Melvo. A similarity appears, therefore, between the performance of the fat and the performance of the batter in that less aeration occurred when an emulsifier was present.

The addition of an emulsifier is here again credited with the production of the better cake. As this cannot be attributed to increased aeration (25, p. 190), possibly it is connected with the different type of fat dispersal which is described above.

Evaluation of the Freeze-drying Method for Study of Batters and Doughs

It appears to be possible, by employing the techniques herein described, to prepare and examine cross sections of unbaked cake batter. Processing in the freeze-drying apparatus apparently does not disturb the structural pattern of the fat. It is therefore possible to determine differences in the microscopic appearance of fats and their location with reference to other ingredients.

The problem of osmic acid penetration into batter samples prepared with Crisco should be pursued further or another method of fixing the fat applied. The effect of freeze-drying on ingredients other than fat in batters and doughs should be investigated.

If, when this method of studying batters and doughs has been critically checked, and it appears to be sound and to supply information not available by methods commonly used in the past, it can and should be recommended.

CHAPTER V

SUMMARY AND CONCLUSIONS

A method of studying batters and doughs has been developed which is believed to be superior to any previously used. For the first time, it is believed, it has been possible to view a true cross section of unbaked cake batter microscopically. Distortion and artifacts are believed to have been held to a minimum.

Extensive preliminary work was conducted to determine how best to apply the freeze-drying principle to the study of batters and doughs. Various methods of obtaining and freezing samples for dehydration were tried. Time and temperature for optimum dehydration by freeze-drying were studied carefully. Dehydration with alcohols at low temperatures was also attempted, although this procedure was soon abandoned as unsatisfactory. Considerable effort was expended to obtain complete fixation of fat with osmic acid. The method of introduction as well as time and temperature were given careful consideration.

Two samples of fat were employed, one containing an emulsifier (Crisco) and the other none (Melvo). Samples of cake batters made with each fat were dehydrated by freeze-drying and samples of whipped fats were processed simultaneously. The fat was fixed with osmic acid vapors

at 60°C. (430°F.) and the samples embedded in paraffin.

From the embedded material permanent slides were prepared and examined microscopically. Photomicrographs were taken to facilitate examination and comparison.

By microscopic examination, intercellular spaces of whipped Crisco appeared to be slightly thicker and the air cells slightly larger than those noted for whipped Melvo. This was associated with easier sectioning of the Melvo samples. The major significance of the portion of the study dealing with whipped fat was that it was found possible to process the sample in the freeze-drying apparatus, and after fixing with osmic acid, embed it in paraffin at 65°C. (149°F.) and remove the paraffin with xylol without disrupting the aerated structure.

The cake batter was found to be a dispersion of air cells, starch granules and fat in a matrix of hydrophilic constituents. The fat and starch frequently appeared as part of the wall of the air cell. The fat sometimes partially surrounded the cell, either as a continuous segment or as a row of globules, depending upon whether an emulsifier was present in the fat.

Organoleptic characteristics of baked cakes prepared with each fat were considered in relation to the dispersion of air in the whipped fats. Although less air was incorporated in the whipped Crisco, this fat produced a

lighter, more tender cake with larger volume. Possibly the production of the better cake is partially due to the different type of fat dispersal that exists when Crisco rather than Melvo is used.

Recognizing the necessity of checking and possible modification, it appears that this method makes a contribution to the microscopic study of batters and doughs. Thus it may be indirectly related to the interests of homemakers and the food industry.

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