The Freeze Fracture Ultrastructure of Peanut Oil and Other Natural and Synthetic Triacylglycerol Droplets

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ABSTRACT

The freeze fracture morphology of some emulsified natural and synthetic triacylglycerol oils was examined. Emulsions were frozen by two methods, immersion in liquid melted Freon 22 (rate ~ 100 K/sec) or by a jet of liquefied propane (rate ~ 10,000 K/sec). Emulsion droplets appeared spherical regardless of freezing method. Droplets frozen with propane appeared featureless in cross-fracture and demonstrated smooth cores regardless of oil type. Those frozen by immersion possessed cores exhibiting lamellae embedded in an amorphous matrix. Pure unsaturated oils such as triolein appeared structureless regardless of freezing rate, whereas natural oils exhibited characteristic morphologies which were partially related to their saturated fatty acid content. Immersion frozen peanut oil possessed, in addition to interior laminations, distinct surface laminations regardless of droplet size, emulsion preparation technique or buffer pH. The laminations were 100 Å thick and extended 3-15 layers deep into the droplet and were caused by long chain C_{20}, C_{22} and C_{24} fatty acids. Six kinds of peanut oil were examined and their droplet surface laminations could be grouped into three structural classes. There was no correlation between structural features of the peanut oils and their atherogenicity. The type of surface lamination that a peanut oil exhibited appeared to be related to its ratio of oleic to linoleic acid. The cryoprotectant glycerol was soluble in olive oil to < 0.1% and produced no morphological alterations of the droplet.

INTRODUCTION

Compared to crystallized fats, the molecular structure of liquid oils is poorly understood. Triacylglycerols and other long-chain lipids exhibit a variety of crystal structures (polymorphism) depending on their composition and the way they are solidified (1). Unlike water, which expands to almost 110% of its liquid volume when frozen, long-chain lipids contract to ca. 90% of their liquid volume when crystallized (1). The often numerous, sharp X-ray diffraction bands that are seen with solid lipids are replaced by one or two diffuse halos when the lipid is melted. N-parraffins all exhibit the same X-ray diffraction halo which is thought to represent the lateral separation of 4.6 Å between the hydrocarbon chains (2). In addition to the 4.6 Å peak, liquid long-chain alcohols and fatty acids exhibit another smaller peak that shifts with the number of carbon atoms in the chain and is thought to correspond to the length of the molecule (3,4). According to this, long-chain molecules in the crystalline state are ca. 15% longer than in the liquid state (3). From X-ray diffraction studies, Stewart and others have postulated that the orientation of molecules is not a system of randomly twisted and tangled chains. Instead, a specific space arrangement is postulated whereby for any one molecule the nearest neighbor is roughly straight and parallel (3-6). Swarms of molecules in parallel array are thought to exist in long-chain liquids. Vandenheuval has calculated a minimum swarm dimension for 1-undecanol of 30 Å (500-1000 molecules) (5). And Larsson has proposed that order extends over ca. 200 Å (7) in liquid triacylglycerol. Crystalline triacylglycerols adopt a tuning fork conformation (8) and Larsson (7) and Callaghan (9) have provided evidence that the tuning fork structure also exists for triacylglycerols in the melt.

Interest in the microscopy of liquid triacylglycerol droplets has been stimulated by the observation that lipases produce visible liquid crystalline product phases on the surface of droplets during hydrolysis (10,11) and although membranes and liquid crystalline systems have been extensively probed by the freeze fracture technique, there are few studies directed specifically at fat droplet structure. The freeze fracture technique is ideal for studying fat droplets and lipid-water systems because there is no dehydration which may extract lipid as well as water. Buchheim and coworkers have studied the freeze fracture morphology of fat droplets in the production of butter from cream (12,13). The phase behavior and polymorphism of bovine milk fat can be understood as the sum of the behavior of three groups of glycerides, which act as independent solid solutions (14). The high and medium melting fractions which together make up 30% of the total are solid at temperatures below 35 C (14). Thus milk fat quickly becomes a mixture of liquid and solid triacylglycerols upon exposure to room temperatures. Buchheim's freeze fracture micrographs of butter fat show mixtures of smooth spherical droplets, deformed crystallized particles, and particles exhibiting smooth and crystallized regions (12). To our knowledge there are no comparative studies of plant oils by the freeze fracture technique.

The following study was made as a prelude to in vivo studies of intestinal fat digestion. In order to follow the hydrolysis of fat droplets in the intestinal lumen by freeze fracture microscopy, fat droplets with a distinctive morphology were sought which would not be confused with other nonlipid particles of the same shape and morphology. The purpose of this study was to examine the freeze fracture appearance of naturally occurring and synthetic triacylglycerol droplets and to attempt to relate the observed morphologies to droplet composition.

MATERIALS AND METHODS

Oils

The pure triacylglycerols trilaurin (12:0), triolein (18:1), trilinolein (18:2), and trilinolenin (18:3) and the natural triacylglycerol oils coconut, sesame, corn, soybean and cottonseed were purchased from Sigma Chemical Co. (St. Louis, MO). Peanut, sunflower, safflower and olive oil were obtained from local grocers. African peanut oil (APNO), South American (SAPNO), cold-pressed (CP), randomized (RPNO), and a simulated reconstituted peanut oil (PGFR) were all gifts generously donated by David Kritchevsky of the Wistar Institute (Philadelphia, PA).

Oils were checked for purity on silica gel 60 thin layer chromatographic (TLC) plates (E. Merck, Darmstat, Germany) in a solvent system of 100:1 diethyl ether/NH_4OH and judged to be 99% pure or better. Highly purified peanut oil was prepared on a column of silicic acid (100-200 mesh) (BioRad Labs, Richmond, CA) according to Nevenzel (15). Uniformly labeled [^{14}C] glycerol (171 mCi/mmol) from

Amersham (Arlington Heights, IL) was used without further purification. All solvents were pesticide grade or better.

Emulsion Preparation Freeze Fracture and Fractional Crystallization

Emulsions were prepared by sonicating with a W-105D sonifer cell disruptor (Heat Systems Ultrasonics, Planview, NY) at 55 W for 15 sec, a mixture of 10% gum arabic (Sigma) in 40 mM Tris base buffer (pH 7.00) containing 150 mM NaCl, 1 mM CaCl and 0.02% NaN_3 in a ratio of 2:1 (v/v) with the sample oil. Oils which were solids at room temperature were warmed above their melting point prior to sonication. For fractional crystallization studies, a sample of purified peanut oil was subjected to centrifugation in a Beckman RC-2 refrigerated ultracentrifuge at -10 C for 10 min at 10,000 rpm. This procedure produced a fractional crystallization of the oil and yielded a clear upper fraction and an opaque phase in the lower portion of the centrifuge tube.

A modified procedure by Moor (16) was utilized for freeze fracture and included the utilization of flat gold planchets (Balzers Union) roughed with sandpaper to allow for increased sample adhesion. Emulsions were prepared with 30% glycerol and without cryoprotectant, and the sample volume used was 0.5 µL. Immersion freezing was accomplished by immersing planchets with sample in melted Freon 22 (-160 C). Slow cooled samples were produced by placing samples on planchets in a conventional freezer at -10 C on a solid brass block. After 1 hr, samples were immersed in melted Freon. To assess the effects of rapid freezing, samples were placed between two flat copper planchets and frozen with a jet of liquified propane (-165 C) in a Balzers QFD 101 Cryojet device. Each oil was freeze fractured at least 3 times. Fracturing was done in a Balzers 360 M freeze etch plant at -120 C and 10^{-7} Torr. The replica was produced by coating with 20 Å of platinum and 200 Å carbon.

The procedure of Bordi (17) for parlodion coating and cleaning of highly fragile replicas was used with some modifications; replicas were floated off in 10% methanol and immediately immersed in N-N-dimethylformamide (E. Merck) for two 20-min steps to remove all traces of lipids. The final cleaning step utilized 50% (conc) HCl for 1 hr. Replicas were mounted on 200 mesh hexagonal grids and viewed in a Philips 200 electron microscope. Droplet diameter was measured according to van Venetie (18).

Fatty Acid Analysis and Effect of Sonications

Pancreatic lipase (triacylglycerol hydrolase E.C. 3.1.1.3) (Calbiochem, La Jolla, CA) was partially purified on Sephadex G-75 and found to be free of colipase activity. It was used to study the composition of the exterior layers of peanut oil. An aliquot of the prepared emulsion was incubated with pancreatic lipase (1500 Tributyrin units/mL) in 50 mM Tris buffer (50 mM Tris Base + 150 mM NaCl + 1 mM $CaCl_2$, pH 7.60) at 23 C under constant agitation at pH 7.02. Samples from the reaction vessel were removed at 1,3,5,8 and 12-min intervals as the reaction proceeded. Products were acidified, extracted in ethyl ether (EE) and separated on TLC plates in the system 100:1 EE/NH_4OH. Monoacylglycerol (MG), diacylglycerols (DG) and fatty acids were visualized with iodine vapor, scraped off and methylated according to Metcalf (19). These samples were analyzed by gas liquid chromatography on an SP2100 methyl silicon capillary column in a Hewlett Packard 5840 gas chromatograph equipped with a flame ionization detector and 5840 A Hewlett Packard electronic integrator. Time of sonication and the effect of the presence or absence of O_2 was examined to determine if any hydrolysis products were produced during emulsion preparation. Emulsions were produced by vortexing and sonicating the oils for intervals of 5, 20 and 40 sec in the presence of air or under continuous nitrogen bubbling. Emulsions were extracted in ethyl ether and prepared as above for gas chromatography.

Water/Triglyceride Partitioning for Glycerol

2.65 nmoles of [^{14}C] glycerol were added to 200 µL of buffer (150 mM NaCl + 1 mM $CaCl_2$ + 50 mM Tris Base, pH 8.30) containing 0.66% gum arabic and 4.10 M glycerol (30%). The control contained the same amount of radiolabeled glycerol in 200 µL of buffer with gum arabic to give a final glycerol concentration of 0.012%. The final radioactivity of each mixture was 500 dpm/µL. Each of these solutions was overload with 200 µL of olive oil, agitated (280 strokes/min) at 23 C for 18 hr, then centrifuged at 15,600 x g for 5 min. Aliquots of 100 µL were taken from each phase and assayed for activity.

RESULTS

The size frequency distribution curve for a sonicated gum arabic stabilized triacylglycerol emulsion is shown in Figure 1. The diameter of cross-fractured particles was measured (range 0.05-3.33 µm) and a standard size frequency curve was generated using a method by Hennig and Elais (20).

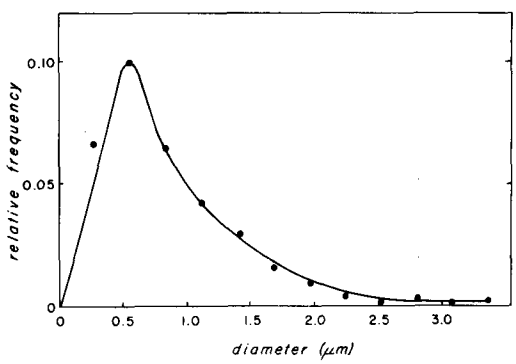

FIG. 1. Standard size frequency distribution for triaclyglycerol emulsions stabilized by 10% gum arabic used in this study.

Figures 2 and 3 show cross-fractured droplets from 8 oil types, all of which were composed primarily of esters of 16 and 18 carbon fatty acids (FA). Frozen droplets exhibited a spherical configuration free of distortion regardless of droplet size or type. When frozen by immersion they exhibited two regions with structural features, the surface and the core. Table I shows the fatty acid composition of the oils used in this study arranged according to increasing percent saturation so that comparisons may be drawn between oil composition and oil droplet morphology. Water/triglyceride partition coefficients for glycerol were measured at two glycerol concentrations, 0.012% and 30%, and found to be 1314 and 1184, respectively, corresponding to a solubility of < 0.1% in olive oil. When frozen by immersion, pure unsaturated monoacid triacylglycerols such as triolein (18:1), trilinolein (18:2) and trilinolenin (18:3) produced droplets which appeared featureless in cross-fracture. The surface of such a droplet was smooth, as was the core (Fig. 2). The micrographs in Figures 2 and 3 were arranged in order of increasing density of core and surface characteristics. Surface characteristics were manifested in the form of

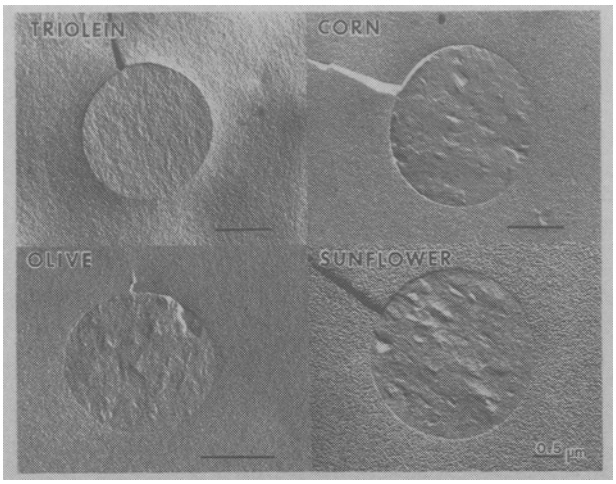

FIG. 2. Cross-fractures of oil droplets frozen by immersion, composed primarily of esters of 18 carbon fatty acids arranged according to increasing degree of morphological complexity. Triolein, corn, olive, sunflower seed.

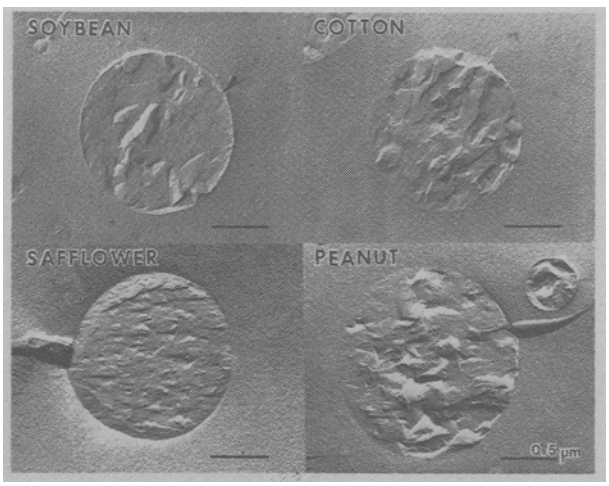

FIG. 3. Cross-fractures of oil droplets frozen by immersion, composed primarily of esters of 18 carbon fatty acids arranged according to increasing degree of morphological complexity. Soybean, cottonseed, safflower seed and peanut oil.

lamellae (peanut, Fig. 3) whereas core characteristics were exemplified by long or short "wavey" lamellae which existed singly as plates (corn, Fig. 2) or in layers of plates (peanut, Fig. 3).

Although the morphologies of each type of oil droplet were diagnostic for oils frozen by immersion, there were no obvious correlations of structure with degree of saturation. Corn oil was 15% saturated but contained smoother interiors than safflower oil which was ~ 6% saturated. Both safflower and olive oils contained ca. 6-7% saturated acids, yet safflower exhibited more interior structure. Olive oil contains ~ 84% trioleylglycerol. Sunflower, soybean and sesame seed oil all appeared similar in cross-fracture. Of these three oils, soybean contained the highest amount of 18:2 (50.7%) while sesame contained the largest percentage of 18:1 (45.4). Cotton contained the largest amount of 16:0 (23.4%) among all the oils in Table I and produced droplets with cores which were similar in complexity to peanut oil. Peanut oil will be described later in more detail.

In order to assess the effect of freezing rate on triglyceride morphology, the oils were frozen rapidly in a jet of lique-

TABLE I

Fatty Acid Compositions of the Plant Oils That were Used in this Study

Oil type	(Weight %)											% Saturation	Melting point (C)	18:1/18:2
	12:0[a]	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	24:0			
Safflower	–	–	6.0	–	–	18.6	70.1	3.4	–	–	–	–	6.0	?	
Sunflower	–	–	5.6	–	2.2	25.1	66.2	–	0.9	–	–	–	8.7	17.0-18.0	
Olive	–	trace[b]	6.9	–	2.3	84.4	4.6	–	0.1	–	–	–	9.3	-6.0	
Soybean	0.2	0.1	9.8	0.4	2.4	28.9	50.7	6.5	0.9	–	–	–	13.4	-16.0	
Sesame	–	–	9.1	–	4.3	45.4	40.4	–	0.8	–	–	–	14.2	-6.0	
Corn	–	1.4	10.2	1.5	3.0	49.6	34.3	–	–	–	–	–	14.6	-20.0	
Peanut (American)	–	–	11.2	–	2.5	47.8	30.8	–	1.3	1.4	3.2	1.7	19.9	3.0	1.55
Peanut (cold-pressed)	–	–	11.1	–	2.5	50.8	29.9	–	1.1	1.3	2.2	1.1	19.8	3.0	1.70
Peanut (African)	–	trace	9.4	–	3.1	58.6	21.7	0.3	1.3	1.2	2.7	1.3	17.8	3.0	2.70
Peanut (South American)	trace	trace	10.9	–	3.2	36.4	41.1	0.9	1.4	1.2	3.2	1.4	20.1	3.0	0.90
Peanut (randomized – interesterified)	–	–	11.5	0.1	2.7	50.9	27.9	–	1.4	1.3	2.6	1.1	19.3	3.0	1.82
Peanut (PGFR)	–	–	10.5	0.56	2.2	42.3	39.1	0.34	1.3	0.32	3.0	0.32	17.3	3.0	1.10
Cotton	–	1.4	23.4	2.0	1.1	22.9	47.8	–	1.3	0.2	–	–	27.2	-1.0	
Coconut[c]	45.4	18.0	10.5	0.4	2.3	7.5	trace	–	–	–	–	–	92.1	25.1	

[a]Carbon chain length: number of double bonds.
[b]Trace > 0.1%. c
[c]8.4% - 10:0; 5.4% - 8:0; and 0.8% - 6:0.

fied propane either with (30%) or without glycerol. A composite peanut oil droplet is shown in Figure 4. The upper portion of the micrograph illustrates the morphology seen upon immersion freezing and the lower portion shows that all surface and core characteristics disappear when the oil is jet frozen. All oils in Figures 2 and 3 demonstrated smooth cores and surfaces when frozen in this manner. Only randomized and PGFR peanut oils showed a small percentage (≤ 5%) of laminated droplets, but all had smooth cores. No morphological differences were observed between any samples frozen with or without glycerol.

In order to investigate the effect of a small percentage of a saturated short-chain triglyceride on the freeze fracture morphology of unsaturated trioleoylglycerol, trilaurin (2-10%) was dissolved in triolein (Fig. 5). This small amount of trilaurin also simulated the average saturation content of some of the natural oils in Table I. When frozen by immersion, droplets of pure trilaurin solidify into masses of planar crystals arranged in layers (similar to Fig. 5D), but when added to pure unsaturated triolein, trilaurin induced no structural characteristics at concentrations of 2 or 10% (Fig. 5A and B). Only when the droplets were initially cooled to -10 C did large crystalline aggregates appear in place of round smooth droplets (Fig. 2C and D). Since lauric acid is considerably shorter than the component acids of the oils in Figures 2 and 3, lauric acid-rich coconut oil was examined. Regardless of the freezing method, coconut oil droplets possessed a finely textured core (Fig. 6). This oil is composed primarily of saturated triglycerides (92.1%) with ca.

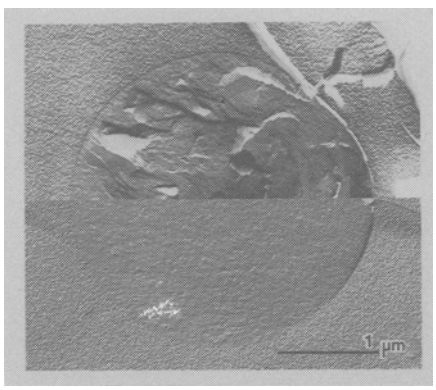

FIG. 4. The effects of immersion freezing and jet freezing on triglyceride morphology. The composite shows a droplet of highly structured peanut oil (upper half of micrograph) after immersion freezing. All structure is lost when droplets are frozen in a jet of liquid propane (lower half).

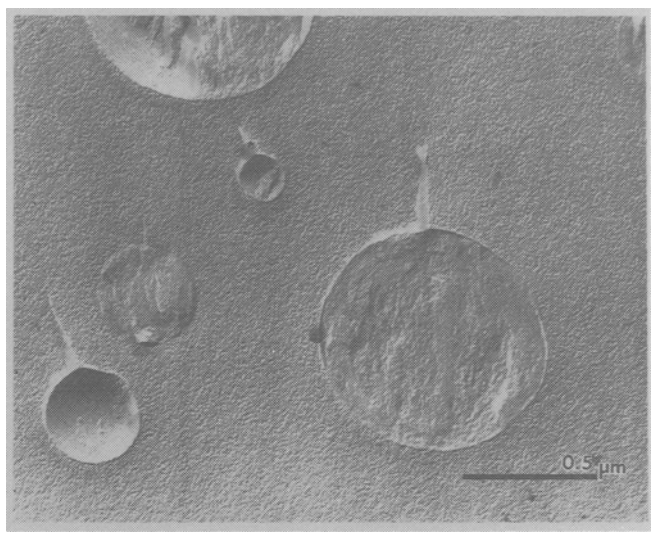

FIG. 6. Immersion frozen droplets of coconut oil composed primarily of saturated triglycerides (92.1%).

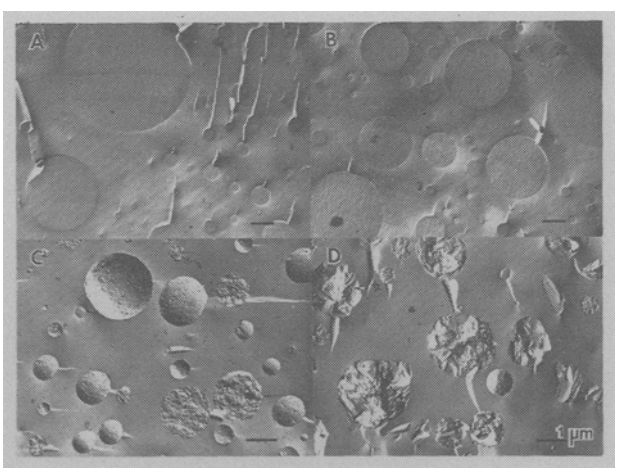

FIG. 5. The effect of a saturated short-chain triglyceride on the morphology of slow cooled-immersion frozen unsaturated trioleylglycerol. (A) 2% Trilaurin (12:0). (B) 4% Trilaurin rapidly frozen by immersion in Freon. (C) 2% Trilaurin. (D) 4% Trilaurin cooled to -10 C for 1 hr then immersed in melted Freon 22.

13% short-chain FA (6:0, 8:0, 10:0). Lauric acid comprises 45.4% of the total FA species of coconut oil and only 9.8% of the FA are 18 carbon types, which distinguishes coconut from all the other oils in Table I. Since it is in a semisolid state at room temperature (mp 25.1 C), one would expect its droplet morphology to be a complex array of plates and aggregates, but the inclusion of such a large proportion of lauric acid along with a majority of saturated species did not produce any striking internal droplet features (Fig. 6).

The only oil to possess very long chain FA (22:0, 24:0) was peanut (PNO). It was also the most distinct morphologically due to the surface laminations it possessed when frozen by immersion (Fig. 7). The appearance of the laminations remained unchanged regardless of pH, time of sonication, and presence or absence of O_2 during sonication. The number of surface laminations varied from 3-15 deep per droplet and each measured ~ 100 Å in thickness. Figure 7 shows the appearance of six types of PNO in order of increasing degree of surface laminations. They may be catagorized according to three classes of surface structure based on the straightness of a fracture edge. The first class is distingushed by short curved edges and includes African and randomized PNO (Fig. 7A and B). The next class possessed long straight fracture edges stretching from pole to pole and includes American and cold-pressed PNO (Fig. 7C and D). The third class was characterized by short straight "arrowhead" type fractures with the tips of the arrows pointing toward the

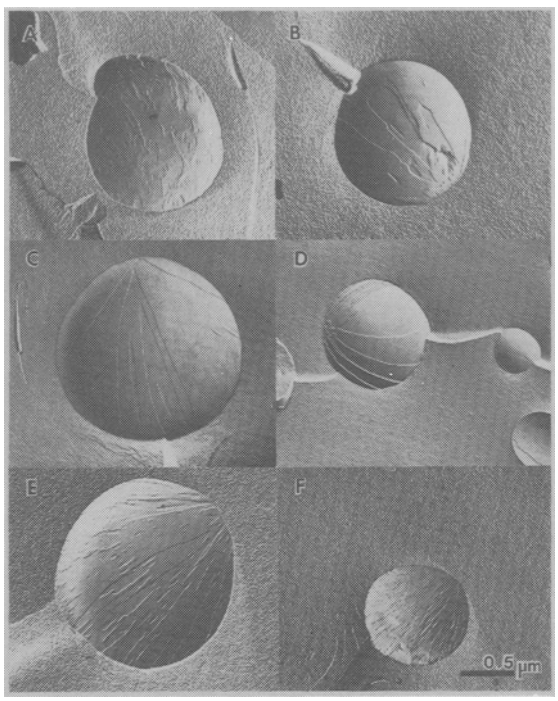

FIG. 7. Six types of immersion frozen peanut oil arranged in order of increasing degree of surface laminations. A-F African, randomized, cold-pressed, North American, PGFR and South American.

droplets equator. South American PNO and the reconstituted oil designated PGFR were included in the third class (Fig. 7E and F).

Neither the percent saturation nor the total percentage of all long-chain FA (20 carbons or greater) correlated with the type of surface laminations for PNO frozen by immersion. The South American oil possessed the most complex surface fracture morphology, 20.1% saturation and a total of 7.2% long-chain FA. PGFR possessed surface morphology similar to South American PNO, was 17.3% saturated and contained 5.0% total very long chain FA. The only ratio that did correlate with the type of laminations was the oleic: linoleic ratio. Those oils with a ratio of 1.1 or less resembled South American PNO, those with a ratio averaging 1.7 were similar to the American oil while oils with a ratio higher than 1.7 resembled African PNO (ratio 2.7). Since PGFR contained 3-4 times less 22:1 and 24:0 and was similar to South American PNO in appearance, the 20:0 and the 22:0 fatty acids may be instrumental in producing the consistent surface laminations on PNO.

The nature of the unique morphology of PNO was further investigated by addition of unsaturated triolein, fractional crystallization and digestion by pancreatic lipase. Triolein was added to purified PNO in the ratios of 25, 50 and 75% (w/w) and the appearance of the droplets after immersion freezing was examined (micrographs not shown). At 25% trioleylglycerol, the appearance of PNO droplets remained unchanged. At 50% triolein, surface laminations disappeared and the core appeared smooth with a few long flat laminations. At 75% triolein, all surface and core structure was eliminated and droplets ultrastructurally resembled pure triolein in appearance (Fig. 2A). The total percentage decrease of long-chain saturated FA due to dilution by triolein was from 5.8 in the intact oil to 3.1 in the 50:50 triolein/PNO mixture.

Purified American PNO was then centrifuged at -10 C for 10 min at 10,000 rpm and two separate phases were produced, one clear, the other opaque. When sonicated in the presence of 10% gum arabic and buffer and then frozen by immersion, each phase produced a distinct droplet type which was reproducible (Fig. 8). The clear low-melting phase produced droplets which were practically devoid of exterior laminations but the cores were full of large polymorphic crystals (Fig. 8B). Droplets from the opaque high-melting phase possessed the opposite characteristics, (Fig. 8A), smooth cores and heavily laminated surfaces (up to 30% of the drop volume). Table II shows the fatty acid composition of the separate phases. When comparing the intact, opaque and clear phases, relatively little difference was seen in the percentages of the major FA (16:0, 18:1, 18:2); however, the percent saturation of the low melting clear phase (17.34) was significantly different from the intact oil (19.94). This difference was mainly due to the 2.5% decrease in the amount of 20, 22 and 24 carbon fatty acid species in the clear oil phase. This again is evidence that surface laminations in peanut oils are caused by their very long chain fatty acid content.

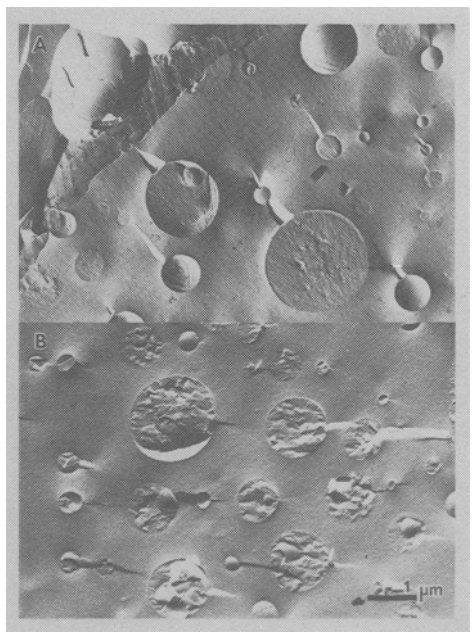

FIG. 8. Fractionally crystallized peanut oil droplets frozen by immersion. A shows droplets produced from the opaque phase exhibiting numerous surface laminations and smooth cores. B shows droplets produced from the clear phase with the opposite characteristics, smooth surfaces and rough cores.

Finally, partial hydrolysis of PNO by pancreatic lipase was performed over time (Fig. 9) and the products (FA, monoacylglycerols, diacylglycerols) were analyzed by GLC. If the composition of the surface laminations at 23 C differed from that of the core (intact oil), the products of the interfacial action of pancreatic lipase might bear this out. An emulsion of American PNO was incubated with lipase for up to 12 min under constant agitation at pH 7.60. Reaction products were removed at intervals, separated on TLC, and analyzed by GLC. After 1 min ca. 4% hydrolysis of the oil had taken place and at 12 min over 40% hydrolysis occurred. If the outer laminations were enriched in saturated FA, then the early reaction products should have been more saturated than the later reaction products. The experiment was exhaustively run (6 times) and at no time were

TABLE II

Gas Chromatography of Fractionally Crystallized Purified Peanut Oil

FFA	Intact	S Phase	L Phase
	% Intact	% Opaque (semisolid)	(% Liquid phase)
16:0	11.24	12.11	11.25
18:2	30.82	32.33	32.35
18:1	47.81	47.22	49.12
18:0	2.53	2.50	2.17
20:1	1.42	1.16	1.18
20:0	1.29	1.08	0.85
22:0	3.18	2.47	2.04
24:0	1.70	1.13	1.03
Unsaturation[a] ratio	19.94	19.29	17.34
Freeze fracture morphology	numerous surface laminations – rough core	numerous surface laminations – smooth core	1-2 surface laminations – rough core
% Long chain FA[b]	7.56	5.84	5.10

[a]% unsat/% sat.
[b](>18:0).

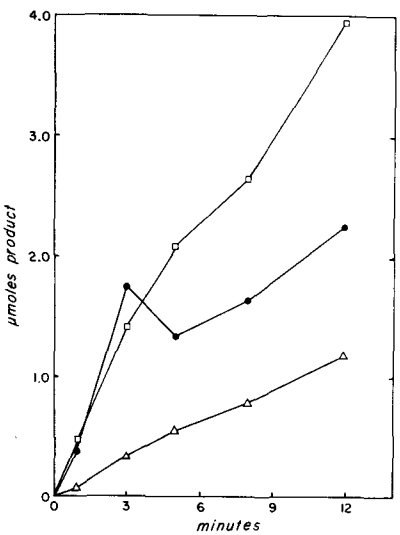

FIG. 9. Monoacylglycerols, fatty acids, and diacylglycerols produced by the hydrolysis of peanut oil at pH 7.60 with pancreatic lipase (1500 tributyrin units/mL). Fatty acids □—□, diacylglycerols ●—●, monoacylglycerols △—△.

the early products more saturated than the later. The total μg of FA liberated during hydrolysis and calculated μg of FA taken from the equation 2 MG + DG/2 agreed within 10%. The calculated composition of PNO (from hydrolysis products) was the same as the GLC composition of the bulk oil. These results suggest that the composition of the surface and the core of PNO droplets was similar and that the organized surface layers occurred during immersion freezing. Figure 4 shows the smooth morphology of the jet frozen droplet which supports the lipase data.

DISCUSSION

The results of this study clearly show that immersion cooling to -160 C of a variety of liquid triacylglycerol droplets cannot entirely prevent phase transitions. When ordered laminations were seen, they were reproducible and exhibited curved and wavy forms which suggest a liquid crystalline structure (13). Freezing in a jet of liquid propane preserved the liquid state in many of the oils. This is in agreement with Berger's study of nonemulsified fats (21) where the liquid components of the fat appeared to be set into an "amorphous glass." Although the temperature at which crystallization starts in emulsified systems is lower than in nonemulsified systems (22), trioleylglycerol, which normally solidifies at -4 C, still showed no signs of crystallization after cooling to -160 C by either freezing method. A "glass-like" packing has also been seen in the hydrocarbon chains of frozen Lα and Hα phases of phospholipids. Upon freezing (-160 C), both phases showed a shift of their characteristic 4.5 Å X-ray diffraction band to a more condensed but still disordered 4.2 Å broad reflection (23).

The growth of crystals in any frozen preparation is a function of the samples freezing velocity (24). The objective of freeze fracture is to minimize the crystal size by cooling the specimen at the fastest possible rate. A replica simply reveals the sample's true crystalline structure. Liquid regions are represented by areas which appear "smooth," but in reality these areas are composed of crystals which are smaller than the platinum grain size. Thus the comparatively smooth portions of the droplet interiors that are shown in this study may be a condensed solid amorphous phase that is closer to the structure of the liquid state than the crystallized state.

But what about the swarms of parallel arrayed molecules that Larsson (7) and Vandenheval (5) suggest exist in liquid lipids? If in the melt, the tuning fork shaped molecules line up in rows head-to-tail, side-by-side in a rough approximation of the crystal structure then there could be fracture discontinuities at ~ 40 Å intervals (the approximate length of a long-chain triacylglycerol molecule). The resolution available to freeze fracture analysis is limited to the size of the platinum grain in a replica. Ideally, a 20 Å thick layer of platinum is laid down on a flat smooth fracture face, but local surface irregularities produce areas of deficiencies or accumulations. Although we thought we could see some tiny parallel lines in the "amorphous liquid" regions of the fractured droplets, there were often tiny parallel lines in the

aqueous regions of the replica too. Thus, although in our hands, freeze fracture microscopy did not provide any indication of swarms of lipid molecules, the replica coating may have covered such evidence.

Berger et al. (21) suggest that during freeze fracture, the liquid components of fat are set into an amorphous glass and that only those crystals that were present in the uncooled specimen appear in the replica. We have proven that the laminations seen in certain oils after immersion freezing do not exist in the droplets at room temperature (23 C) by a number of methods. Polarizing light microscopy of the droplets showed no birefringence and the oils appeared to be optically clear before freezing. When the oils were jet frozen, all crystalline morphology was eliminated. In addition, the lipase experiments which suggested that there were no significant differences between the outer and inner regions of peanut oil droplets, also lend support to the idea that the laminations were formed during immersion freezing. With the exception of peanut oil, the ordered structure of the immersion frozen plant oils, when present, appeared scattered within the core of the droplet. Since triolein showed no ordered structure in this region, the laminations seen in the natural oils must have been caused by the aggregation of their saturated fatty acids (nucleation) during immersion freezing. The loss of laminations in peanut oil droplets that occurred when they were diluted with triolein suggest that there are "seed glycerides" in peanut oil which induce ordered arrays in otherwise formless lipid. This also suggests that the composition of the laminations is distinct from the smooth areas.

The degree of saturation of an oil by itself appears to be a poor predictor of structure induced by immersion freezing as shown in Figures 2 and 3. Corn oil which was 14.6% saturated exhibited a low density of internal laminations whereas the slightly more saturated peanut oil (19.9%) exhibited extensive laminations. Safflower oil (6.0% saturated) exhibited as much structure as corn oil. Coconut oil is in a class by itself and the high percentage of medium-chain fatty acids appear to give it a fine grained freeze fracture appearance whether frozen by immersion or in a propane jet (Fig. 6). So even though each natural oil droplet exhibited a unique freeze fracture appearance, the percentage of saturated fatty acids in a given oil can only partially predict the type of morphology that will appear following immersion freezing. Oils containing saturated long-chain fatty acids will exhibit distinct morphological characteristics.

In this study peanut oil stood out because of its extensive surface laminations (Fig. 7). Several pieces of evidence indicate that these laminations are caused by the very long chain saturated fatty acids in peanut oil (20:0, 22:0, 24:0). First, cottonseed oil, which is even more saturated than peanut but does not contain significant long-chain saturates, did not exhibit extensive surface laminations. Second, when a synthetic peanut oil (PGFR) was made by adding arachidic (20:0) and behenic (22:0) acids to plant oils that do not exhibit surface laminations (olive, cottonseed and safflower), surface laminations appeared (Fig. 5E). Even after jet freezing, a small percentage of droplets still possessed surface laminations. Finally, the fractional crystallization experiment (Fig. 8) suggested that unsaturated fractions of peanut oil will not exhibit surface laminations. Other evidence that saturated triacylglycerols preferentially orient at the droplet surface comes from studies of the milk fat globule membrane where the acylglycerols associated with the isolated membrane are more saturated than the bulk milk fat (25). In surface science there is much indirect and inferential evidence that surface regions have altered structure and properties relative to the bulk phase (26). Almost all of the immersion frozen oils in this study did show one or two surface layers. Why the peanut oil laminations were so numerous and why the interior laminations of the other oils did not orient at the droplet surface is unknown. It is interesting to note that oil droplets possessing characteristics identical to peanut oil (i.e., laminations) have been seen in yeast (27) and mouse liver cells (28) in earlier studies. These studies utilized glycerol for cryoprotection along with the immersion method.

Peanut oil has attracted attention because it induces atherosclerosis in a number of animal models (29). However, when it is randomized (i.e., when the fatty acids esterified at the three positions of glycerol are chemically rearranged), then peanut oil loses much of its atherogenicity (30). The theory now is that the long-chain saturated acids are involved in the atherogenicity of peanut oil but only when they are esterified to glycerol with specific combinations of the unsaturated peanut oil fatty acids (31). The peanut oils examined in this study in order of decreasing atherogenicity are South American, African, North American (the cold-pressed was North American), PGFR, and randomized (29-31). Although the 3 classes of surface lamination that were seen in these oils (Fig. 7) did not appear to be related to their degree of atherogenicity, there was a correlation between lamination morphology and the oleic acid/linoleic acid ratio. Those oils with low ratios exhibited the "arrowhead" discontinuities, while those with the high ratios showed uneven fractures through the surface laminations. Perhaps the increased number of breaks in the laminations of the high linoleic acid oils occur because the laminations are weaker and more liquid than in those peanut oils with the lower concentrations of linoleic acid. Clearly, peanut oil will be a marvelous oil for future microscopic studies.

Many peanut cultivars exist with different fatty acid compositions (32) and within a single oil (African) the proportions of 84 isomers have been calculated (33). The very long chain saturated fatty acids (20:0, 22:0, 24:0) are implicated in the formation of surface laminations as shown in this study and as Kritchevsky and others have shown, in the etiology of atherosclerosis in animal models (29). The calculated solubilities of arachidic (20:0) and behenic acids (22:0) at 37 C in triolein are 1.47% and 0.78%, respectively (34). Since they exist in higher amounts in an esterified form in peanut oil, they may crystallize out of the product phases following hydrolysis by lipase. Maybe atherogenic peanut oils will crystallize out while nonatherogenic oils will not. We are currently investigating this possibility.

The freeze fracture morphologies of natural triacylglycerol droplets appear to be species specific and in the case of peanut oils, even cultivar specific if the oils are frozen by immersion. The observed morphologies, which appear to be only partially related to the degree of saturation of the oil, undoubtedly originate from the unique mixture of triglyceride isomers that occur in natural oils.

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Chemical Ionization Mass Spectrometry of Fatty Acids: The Effect of Functional Groups on the CI Spectra[1]

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ABSTRACT

Chemical ionization (CI) mass spectra of functionally substituted fatty esters are a useful aid in determining molecular weight. Isobutane and ammonia CI mass spectra of various hydroxy, keto, epoxy and hydroperoxy fatty esters are reported and discussed.

INTRODUCTION

Although instruments with capabilities for chemical ionization mass spectrometry (CI-MS) have been widely available for a number of years, the literature is surprisingly sparse in systematic studies of CI mass spectra for fatty acids and their derivatives.

CI-MS has been used for the identification of polyunsaturated fatty acids in human serum (1). GC-MS with isobutane as the reagent gas yielded the quasimolecular ion $[M+H]^+$ as the base peak for methyl esters of fatty acids from C_{18} to C_{22} with up to six double bonds. Since the positions of the double bonds could not be deduced from the fragmentation of the underivatized fatty esters, the hydroxylated derivatives were analyzed by CI-MS. Fragments observed were similar to those found in electron impact mass spectrometry (EI-MS) of the same compounds. Suzuki et al. (2) reported CI-MS of methoxy derivatives of esters to locate double bonds in polyene fatty esters. CI-MS spectra of prostaglandins have also been reported (3). Stan and Scheutwinkel-Reich recently reported CI-MS data for the TMS derivatives of hydroxy fatty acids (4–6). They report that CI-MS is better than EI-MS because spectra show both position of OH substitution and molecular weight. Little additional information, however, exists in the literature for fatty acids with combinations of hydroxy, epoxy and/or keto groups in the molecular. We have undertaken a study of the CI mass spectra of these oxygenated fatty acids both as underivatized hydroxy or epoxy esters and as TMS derivatives or solvolyzed epoxide derivatives.

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Ion production by CI consists of reacting the substance under investigation with a known set of reactant ions. This is accomplished by operating the MS ion source at a relatively high pressure (on the order of 1 torr). The reactant ions are formed from the reagent gas by a combination of electron impact (EI) ionization and ion molecule reactions. The compound being analyzed is then chemically ionized within the same ion source by ion-molecule reactions. To suppress EI ionization, the concentration of the same is kept below $\sim$0.1% of the reagent gas concentration. The reactant ions may act with the sample as either Bronsted acids (by proton transfer) or as Lewis acids (by hydride abstraction), forming $(M+H)^+$ and $(M-H)^+$ ions. Additionally, the reactant ions may react with the sample to form a collision-stabilized complex $(M+\text{reactant ion})^+$ (7). These ionic species formed initially may have sufficient energy to further decompose, but the ionic species formed under CI have considerably less energy than the molecular ion formed under EI, and fragmentation is greatly reduced when compared to EI mass spectra. The initial ionic species is formed in a chemical ion-molecule reaction, and the nature of the product formed, its energy distribution, and the resulting fragmentation are jointly determined by the sample and the choice of reagent gas. Both ammonia and isobutane were investigated as reagent gas for fatty acids with various functional groups.

EXPERIMENTAL PROCEDURES

The mass spectra were obtained from a MS-30 mass spectrometer (Kratos Scientific Instruments, Westwood, NJ) equipped with a combined CI/EI source of a model 4535/TSQ quadruple mass spectrometer (Finnigan, MAT, Sunnyvale, CA). EI-MS were produced at 70 eV with a source pressure of approximately 10^{-6} torr on the MS-30, whereas CI-MS were produced at $\sim$150 eV with a pressure measured in the pumping housing arm of the MS 30 of 2×10^{-4} torr.