1985

The Light Microscopy of Triglyceride Digestion

John S. Patton
Russel D. Vetter
Margit Hamosh
Bengt Borgstrom
Mats Lindstrom

See next page for additional authors

Follow this and additional works at: http://digitalcommons.usu.edu/foodmicrostructure

Part of the Food Science Commons

Recommended Citation
Available at: http://digitalcommons.usu.edu/foodmicrostructure/vol4/iss1/5

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Food Structure by an authorized administrator of DigitalCommons@USU. For more information, please contact beckythoms@usu.edu.
The Light Microscopy of Triglyceride Digestion

Authors
John S. Patton, Russel D. Vetter, Margit Hamosh, Bengt Borgstrom, Mats Lindstrom, and Martin C. Carey

This article is available in Food Structure: http://digitalcommons.usu.edu/foodmicrostructure/vol4/iss1/5
During fat digestion (lipolysis) a number of physiochemical events can be seen directly by light microscopy. Hydrolysis of emulsified fat droplets by lipases at pHs above about 6.5 proceeds with the formation of visible product phases that may include both crystalline as well as liquid crystalline phases. The crystalline phase is primarily calcium-fatty acid soap and its formation is favored by high calcium concentrations, alkaline pHs, and inhibited by low pH and monoglycerides. The formation of liquid crystalline product phases are favored by low calcium concentrations, monoglycerides and lipid saturated bile salt solutions. Both phases are solubilized by bile salts but the crystalline phase to a much lesser degree. Colored and fluorescent hydrophobic solutes that are dissolved in long chain triglyceride appear to flow directly into the liquid crystalline product phases where they can be codispersed with the digested lipid by bile salts. Measurements of the shrinking diameters of digesting fat droplets show that enzyme activity on individual droplets falls rapidly during lipolysis. This suggests that lipase molecules are physically displaced from the substrate interface during lipolysis and dispersed in the product phases.

**Abstract**

During fat digestion (lipolysis) a number of physiochemical events can be seen directly by light microscopy. Hydrolysis of emulsified fat droplets by lipases at pHs above about 6.5 proceeds with the formation of visible product phases that may include both crystalline as well as liquid crystalline phases. The crystalline phase is primarily calcium-fatty acid soap and its formation is favored by high calcium concentrations, alkaline pHs, and inhibited by low pH and monoglycerides. The formation of liquid crystalline product phases are favored by low calcium concentrations, monoglycerides and lipid saturated bile salt solutions. Both phases are solubilized by bile salts but the crystalline phase to a much lesser degree. Colored and fluorescent hydrophobic solutes that are dissolved in long chain triglyceride appear to flow directly into the liquid crystalline product phases where they can be codispersed with the digested lipid by bile salts. Measurements of the shrinking diameters of digesting fat droplets show that enzyme activity on individual droplets falls rapidly during lipolysis. This suggests that lipase molecules are physically displaced from the substrate interface during lipolysis and dispersed in the product phases.

**Introduction**

Fats supply more than 40% of the total calories ingested by humans in Western industrial societies (29). Fat digestion and absorption is very efficient (greater than 95%) (6,46), and there is no feed-back regulation to reduce its assimilation. Adult Americans intake of fat averages approximately 150 g/day (29) and this high intake level correlates with increased incidences of atherosclerosis (50) and cancer (31). Gastrointestinal fat assimilation is a process that also enables a vast array of fat soluble (hydrophobic) chemicals to be efficiently absorbed by humans. Among the molecules that are coassimilated with triglycerides during digestion and absorption are some of the essential fat soluble vitamins as well as many hydrophobic xenobiotics, drugs, food additives, carcinogens, and chemicals produced during cooking (41). Commercial fats and oils are often contaminated with small amounts of carcinogens like aflatoxins (17), nitrosamines (25), and polycyclic aromatic hydrocarbons (30). Thus fats and hydrophobic chemicals that are sequestered in fat exert profound effects on human health and disease. These effects are related to their dose, their physicochemical form and their interactions with other lipid molecules with which they are comestabolized.

The lumen of the gastrointestinal tract is anatomically a region that is part of our external environment. Colonized at both ends by billions of microbes and used in the processing of tons of different foods in a lifetime, this dark tube is still largely a "black box" in terms of our understanding of digestion at the molecular level. The chemical and physical disassembly of the exquisite molecular structure of tissues, cells, cell organelles and the varied forms of food including milk is just beginning to be studied (4,11,24,26,51). Although samples of human intestinal content can be obtained with various intubation techniques, following solid test meals (36,38), these samples have rarely been scrutinized by microscopy. Because intestinal contents are heterogeneous mixtures of partially digested food particles, sloughed cells, mucus and numerous other secretions, the classical approach in fat digestion studies has been to chemically characterize the contents following a physicochemical phase separation step by centrifugation (28) or ultrafiltration. In addition to these types of studies a voluminous literature also exists on the biochemistry of the digestive enzymes (41). Thus while considerable chemical information is available, much less is known about microstructural events in digestion.

One way to observe digestion without looking at intestinal
content is to isolate the appropriate digestive juices and expose food particles to them on the stage of the light microscope. This simple approach has been used to study fat digestion (39) and has led to some new insights into the process that chemical studies could not provide. On the stage of a microscope, time, temperature, pH and concentrations of reactants can be controlled. The continual motion of intestinal contents that occurs in vivo, however, cannot be duplicated. This apparent drawback turns out to be the advantage of the technique because once the reactants are combined, physical dispersion which normally accompanies enzymatic events in the gut, is greatly slowed on the unstained microscope slide (39). The present study shows how simple light microscopy has improved our understanding of the lipid phases that can be formed by digested fat and the fate of fat droplet solutes during lipolysis.

**Experimental**

Human and porcine pancreatic lipase and colipase were purified from pancreatic juice or pancreatic extracts as previously described (40,46). Lipase and colipase activities were measured by titration (7) at 23°C on 500 μl tributyrin (TB) in 10 ml buffer containing 2 mM tris (pH 8.3), 1 mM CaCl₂, 150 mM NaCl and 0.02% sodium azide with continuous emulsification obtained by magnetic stirring. For colipase assays, 4 mM sodium taurodeoxycholate (NaTD C) in buffer (7) and a saturating amount of lipase (approx. 10 μg) was used. Lipase and colipase activities are expressed as tributyrin units where one TB unit equals 1 μmol of butyric acid released per minute at pH 8.3 and 23°C. Human duodenal content contains roughly 1000 TB units/ml of lipase and colipase (9).

For the microscopic studies, reactants were usually prepared in four separate solutions and kept on ice. The first solution contained the substrate, either olive oil or triolein emulsified with 10% gum arabic (1:2 oil:gum arabic solution, v:v) and buffered to the desired final pH with Tris or Tris-maleate buffer. The second solution contained the digestive enzymes either as pure pancreatic juice or in a purified or partially purified state. The third solution contained bile salts, either as native porcine bile or as pure NaTD C or sodium taurocholate (NaTC) buffered to the desired pH. In some experiments, a 4th solution containing CaCl₂ was employed. Using a micropipette, desired aliquots of each solution (5–20 μl) were placed as separate drops near to each other on microscope slides. The reaction was initiated by vigorously mixing the tiny drops together with a plastic pipette tip for 5–10 seconds. A timer was started and a coverslip (in most cases sealed with silicone grease to prevent desiccation) was placed over the mixture and the reaction then observed by either a Zeiss or an Olympus Vanox photomicroscope. Photographs were taken with Ektachrome film either ASA 150 tungsten or ASA 400 daylight (for fluorescence). Saturating colors in photographs were obtained by making exposures at 1/4 the maximum light intensity available in the photomicroscopes.

**Results and Discussion**

**Lipolysis at pH 5.0**

Although some dietary fat is partially crystallized at room temperature (10,14), in the stomach most of it will quickly melt into a liquid oil (Figure la). All substrates discussed in this review were oils at 23°C. A heated microscope stage was used to examine reactions at 37°C. In the stomach 10–30% of dietary fat digestion can occur (6,20,23). At least two lipases, lipase from the posterior part of the tongue (21) and gastric lipase from the stomach wall (1,32) initiate fat digestion under the acidic conditions in the stomach. While both of these enzymes appear to be important in normal fat digestion, they are particularly important for fat digestion in the newborn (22), in patients with cystic fibrosis (15,18), and other situations where pancreatic secretion of digestive enzymes is under-developed or impaired. When human milk fat droplets or fat droplets stabilized by gum arabic were partially digested (approx. 10%) by lingual lipase in vitro at pH 5.0 no visible product phases were seen by light or electron microscopy (42). The products of lingual lipase activity, protonated fatty acid and diacylglycerol appeared to remain dissolved in the oil phase of the fat droplet (42,13). Figure 1b shows a schematic illustration of a human milk fat droplet where the lipolysis products remain inside the droplet during hydrolysis by lingual lipase. Buchheim (11), however, has seen "extended lamellar structures" by freeze fracture in the gastric coagulation of pasteurized bovine milk which he attributes to monoglyceride. Since bovine milk fat contains short chain fatty acids, it is unclear whether similar structures would be seen with normal long chain triglyceride. The nature of stomach content remains largely unknown.

The majority of fat digestion occurs in the small intestine through the action of pancreatic lipase and its trypsin-activated cofactor, colipase (9,53). Here undigested triglyceride and the diglyceride produced by lingual lipase are hydrolyzed to yield the final products of fat digestion, fatty acid and 2-monoglyceride. These final digestion products, partly because of their structure and partly because of the physicochemical conditions that exist in the small intestine, will not form, or stay inside of oil droplets (13). Instead they interact with water and cations to form metastable product phases that can be seen with the light microscope (15,39).

In these phases some of the fatty acids are ionized. Although initially all fatty acids produced during lipolysis are protonated, they may secondarily undergo ionization. At pH 8.0 roughly 90% of fatty acids produced by lipase appear to ionize immediately, while at pH 6.0 less than 10% rapidly lose their acidic protons (40). The rate of ionization may be accelerated by cations and bile salts (3,34,47) and the degree of fatty acid ionization can have marked effects on the structure of the product phases. In the absence of bile salts the product phases of fat digestion are relatively stable and will persist for days in a sealed slide preparation. However, in the presence of micellar concentrations of bile salts, product phases may not form. If the sub-microscopic bile salt micelles are preloaded with lipid (i.e., saturated) as occurs when human bile is diluted (12) then the product phases formed during fat digestion cannot be rapidly "dissolved" by the micellar solution. The word dissolved is used loosely here to mean dispersed or solubilized below the resolution limits of the light microscope. But, when the bile salt micelles are empty, (unsaturated) they may rapidly "dissolve" the products of fat digestion (although not always, see below). Thus in preparations with excess bile salts, product phase formation and solubilization occur simultaneously until the micellar solution can no longer solubilize lipid. With excess bile salts, and low calcium concentrations, no product phases are visible in the early stages of lipolysis because products are solubilized as fast as they are produced (Rigler and Patton, in preparation). How the solubilized products of fat digestion are eventually transported across the brush border membrane of the enterocyte is poorly
understood. They may be absorbed either as individual molecules (52) or as aggregates (41) through the fusion of bile salt micelles and liposomes with the brush border membrane.

When human stomach content, containing fat droplets, was mixed with human pancreatic juice and human bile under simulated physiological conditions in vitro two product phases were observed to emanate from fat droplets; first a small amount of crust-like solid crystalline phase formed, then a large amount of isotropic liquid crystalline phase was produced (39). These two phases represent the two general classes of product phase that have been seen in all of our microscope studies of fat digestion.

**The calcium soap phase**

In the presence of free calcium ions, unsaturated bile salt micelles, and physiological concentrations of pancreatic lipase and colipase, a shell of crystalline product phase forms around digesting fat droplets within the first minutes of lipolysis (Figure 2). This crystalline phase is the only phase we have seen that rapidly forms in the presence of unsaturated bile salt micelles.

With small fat droplets this crystalline phase may be the only phase formed (white and black arrows, Figure 2). Larger droplets, however, may produce additional phases (see below). Formation of the crystalline phase exerts water pressure on the larger undigested oil droplets (dyed with β-carotene) which eventually erupt out of the crystalline shells (black arrows, Figure 2) either as one or two large droplets or as many small droplets. The dense crustlike shells sediment with gentle centrifugation and have been shown to be almost pure fatty acid calcium soaps (i.e., one divalent calcium cation bound to two monovalent fatty acid anions) (39). Since 2 monoglycerides comprise up to ½ of the total products of pancreatic lipase hydrolysis, their absence from the calcium soap phase illustrates that calcium soap formation is a secondary precipitation reaction that occurs from the original product mixture of fatty acids and monoglycerides. Although in some cases calcium soap phase may be the only phase observed microscopically (as in Figure 2 with small fat droplets in the presence of excess bile salts), its formation often stops before a reaction is complete and it does not appear to be the dominant product phase under physiological conditions.

At least three factors appear to limit calcium soap formation:

a) Most important is calcium concentration in the reaction medium which may be low or rapidly depleted during the initial stages of the reaction so that none is left to react with fatty acids (39).

b) Calcium soap formation is also limited at acidic pHs. Although the hydrolysis of fat by lipase produces protonated fatty acids, they must then undergo ionization before they can react with calcium. This ionization causes the pH of the medium to drop. In the microenvironment surrounding a fat droplet, rapidly forming calcium soap may quickly lower the local pH and inhibit further soap formation.

c) There is evidence that 2-monoglyceride inhibits calcium soap formation (39). When increasing concentrations of 2-monoglyceride were added to fatty acid oil droplets, calcium soap formation was inhibited. During lipolysis 2-monoglyceride concentrations increase as the intermediate product, diglyceride is hydrolyzed: thus calcium soap formation may be inhibited by monoglycerides as the reaction proceeds.

If all of these factors are operating to inhibit calcium soap formation, why in Figure 2 is calcium soap the only visible phase that forms from small droplets? We do not know for sure but there are several possible explanations. First the small droplets may have been digested before the high calcium concentration (8 mM) in the medium was depleted. In addition, the buffer, 83 mM NaHCO₃ pH 7.5, was probably strong enough to promote fatty acid ionization and keep the pH constant. Moreover, in the presence of unsaturated bile salt micelles, monoglycerides are thought to be solubilized more rapidly than fatty acids (27) which would mean that monoglycerides may have been prevented from accumulating and inhibiting calcium soap formation around the small droplets in Figure 2. Figure 3 shows a photograph from a reaction similar to that shown in Figure 2 but with two notable exceptions. In Figure 3 the pH of the reaction was 6.5 (one unit lower than in Figure 2) and the reaction had proceeded for 20 minutes (compared to 2 minutes in Figure 2). Since substrate and enzyme were not limiting, longer reaction times favor calcium depletion from the medium, and saturation of the bile salt micelles (which means that preferential monoglyceride solubilization by the micelles slows greatly). In addition the lower pH inhibits calcium soap formation. In Figure 3 the second phase (L), an isotropic liquid crystalline phase, can be seen emanating from the β-carotene dyed triglyceride droplet (T). The initial calcium soap phase (C) lies below the digesting fat droplet.

The importance of monoglyceride in the inhibition of calcium soap formation is further illustrated in Figures 4 and 5. Methyleate is a substrate that is slowly hydrolyzed by pancreatic lipase to produce fatty acid (oleic) and methanol. Thus the only lipid product of this reaction is fatty acid. When droplets of methyleate were digested by lipase (Figure 4) they were entirely transformed into crystalline product phase (C). Another example of the importance of monoglyceride is shown in Figure 5 which depicts a droplet of 1.2 diglyceride (D), one of the products of gastric lipolysis, as it was digested by pancreatic lipase. In this reaction lipase produces one molecule of fatty acid and one molecule of monoglyceride per molecule of diglyceride. While this experiment was carried out under conditions similar to those that produced calcium soap with triglyceride as substrate (Figures 2 and 3), only a liquid crystalline phase (L) was produced. These results suggest that calcium soap formation is inhibited when one molecule of monoglyceride is released per fatty acid molecule (compared to one molecule of monoglyceride per two molecules of fatty acid with triglyceride). When diglyceride...
is digested at pH 8.3, Figures 6, 7, and 8 (compared to pH 6.5 in Figure 5) a small amount of crystalline phase (C) forms (Figures 6 and 7). Here it is clearly a secondary precipitation that occurs within the pools of product phase (L). Although both Figures 6 and 7 were taken from the same slide preparation, different crystalline habits were observed in different regions of the slide.

Under physiological conditions calcium soap formation is limited because of low free calcium concentrations, slightly acidic conditions and the presence of partially to fully saturated bile salt micelles. A small amount of crystalline phase (black arrow) occurs at pH 8.0 when native porcine bile and porcine pancreatic juice are used to digest fat (Figure 9). It is interesting in Figure 9 that although the crystalline phase appears to be quantitatively of minor importance relative to the liquid crystalline phases (L and M), yellow bile pigments appear to precipitate with it. Perhaps luminal co-precipitation of certain bile excretory products with fatty acid calcium soaps is a mechanism for prevention of reabsorption. Compared to fatty acids, calcium soaps are poorly solubilized by bile salts (19) and up to 50% of fecal fat has been shown to be calcium soaps in steertrossa, (5) (although it is uncertain whether the calcium soaps found in feces originate directly from pancreatic lipase action). These soaps also make up most of the fecal lipid in infants fed high calcium diets and are responsible for increased fat malabsorption in patients with exocrine pancreatic insufficiency receiving enzymes and calcium carbonate antiacids (19). So, there is much that is unknown about dietary fat—calcium interactions. Clearly the light microscope can be a useful tool for studying these interactions.

Liquid crystalline product phases

In the absence of divalent cations lipolytic products form liquid crystalline product phases. Although bile salts can rapidly solubilize these phases into submicroscopic vesicles and micelles (13) their solubilization capacity is saturable. Thus when liquid crystalline phases are observed to persist in the presence of bile salts it must be assumed that the solubilization capacity of the bile salts is exhausted or nearly exhausted (13; Rigler and Patton, in preparation). At pHs between 6.5 and about 7.0, the dominant liquid crystalline phase that forms during triglyceride hydrolysis appears as an amorphous isotropic pool (L) (Figure 3). Freeze fracture electron microscopy of this phase in the absence of bile salts or in the presence of a saturated micellar solution shows that it has a condensed rough lamellar (i.e., layered) structure (45; Rigler and Patton, in preparation). The relationship between this condensed rough lamellar phase and the cubic or L2 phases (33,34,35) is uncertain. Its water content has not yet been determined. It can be seen in a variety of preparations in Figures 3, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14. Figure 12 shows this phase (L) in a frozen section of fish intestine where a fat meal (T) was undergoing digestion and absorption. It appears to be an important intermediate phase in the intestinal lumen. Chemical analysis of human duodenal content suggests that liquid crystalline product phases are present (48,49). In the presence of an unsaturated micellar solution, liquid crystalline lipolysis products undergo rapid solubilization until the micellar solution is saturated. As the micellar solution nears saturation the initial isotropic phase may be highly swollen with water with variable aqueous spaces between smooth lamellae (Rigler and Patton, in preparation). The isotropic phase (L) in Figure 3 is an example of a water swollen liquid crystalline phase.

At pH above about 7.5 the isotropic phase generated from triglyceride often undergoes a fascinating secondary transformation to form myelin figures (M). These tubular projections of lamellar lipid usually emerge snake-like from the isotropic pools and can be seen to elongate and flow. Their formation can be attributed to additional ionization of the fatty acids in the isotropic pool which causes the phase to further expand its surface interaction with water. Examples of myelin figures (M) that have formed during the digestion of fat at pH 8.0 and above are shown in the presence of natural bile (Figure 9) and in the absence of bile salts (Figure 14). Isotopic phases (L) that are formed during diglyceride (D) hydrolysis (Figures 5–8, 10,11) are similar to those seen with triglyceride, however, the additional proportion of monoglyceride in the diglyceride products enables the isotropic phase to form myelin figures (M) at a lower pH than with triglyceride (Figure 5, pH 6.5). Very vigorous myelin figure formation (M) occurs from the isotropic phase (L) of diglyceride digestion at pH 9.0 (Figure 8).

In addition to the isotropic phase (with its sometimes associated myelin figures), one other distinct liquid crystalline phase has been observed in the absence of bile salts. This phase is transitory and usually appears between the isotropic phase and the digesting oil droplet from which it emanates. It changes within minutes into the isotropic phase and unlike the isotropic phase, it is birefringent by polarizing and Nomorsky light microscopy. Figure 10 shows a ring of this phase (H) as a diglyceride droplet (D) is digested. It appears to move inward (black and white arrows) toward the diglyceride, while on its periphery the isotropic phase appears to dissolve it (black arrows). Figure 11 shows a high magnification of the transition zone between the birefringent phase (H) and the isotropic phase (L). As the birefringent phase dissolves into the isotropic phase, tubular projections (P) appear to flow out into the isotropic phase (black and white arrows). The birefringent phase has also been seen during triglyceride digestion (not shown) and freeze fracture electron microscopy shows it to have a tubular hexagonal structure (45). This birefringent phase may be the liquid crystalline product phase with the lowest water content. The greatest amounts are seen during hydrolysis of larger oil droplets by high concentrations of enzyme where product phase is produced faster than water can penetrate them.

The fate of dissolved solutes in dietary fat during lipolysis

Fat droplets are sinks for a wide variety of beneficial and harmful hydroporphic molecules (i.e., vitamins, sterols, pesticides, xenobiotics, carcinogens, plasticizers, etc.) (41,44). Although it is known that fat can enhance the absorption of many of these exotic hydroporphic molecules (84), the precise manner that it does is poorly understood. A key step appears to be the co-solubilization of lipolytic products and hydroporphic molecules in the bile salt micelle. In the absence of lipolytic product, the micellar solubility of many hydroporphic molecules, particularly crystalline ones like cholesterol (MP 148.5°C) is reduced (12). The liquid fatty acid chains of digested dietary fat make the interior of the bile salt micelle a more favorable environment for many hydrophobic, which means that a greater amount of the hydrophobic can be solubilized, dispersed, and presented to the absorptive membrane.

Colored or fluorescent hydroporphics that are dissolved in long-chain triglyceride can be used as probes of the co-solubilization process that occurs during fat digestion. When crystalline benz(a)pyrene, pyrene, perylene, coronene, β-carotene, Sudan III
The Light Microscopy of Triglyceride Digestion

Captions for Figures 1a to 15 which are presented on two color plates in the following pages.

Figure 1a. Hand shaken emulsion of fat droplets (T) (triolein: 10% gum arabic 1:2 by volume, Normasky Interference optics).

Figure 2. The appearance of β-carotene saturated (approx. 1%) fat droplets (T) after 120 seconds exposure to human lipase and colipase. Undigested fat is extruded (small black arrows) from shells of crystalline calcium soap product phase (C). Undigested fat is not extruded from small particles (black and white arrows). (Total concentrations 8.3 mM CaCl₂, 13.3 mM NaTDC, 83 mM NaHCO₃, pH 7.5, 50 mM NaCl, 1000 tributyrin (TB) units/ml of human pancreatic lipase and colipase, 3.3% olive oil, 37°C bright field).

Figure 3. A second clear liquid crystalline (L) phase appears around a β-carotene saturated fat droplet (T) after 20 minutes exposure to human pancreatic lipase and colipase. (C)-crystalline product phase. (8.3 mM CaCl₂, 13.3 mM NaTDC, 40 mM TriS-maleate, pH 6.5, 150 mM NaCl, 1000 TB units/ml lipase and colipase, 3.3% olive oil, 37°C bright field).

Figure 4. A clear liquid droplet of methyloleate has been entirely transformed into crystalline product phase (C) following 12 hours exposure to human pancreatic lipase and colipase in the presence of calcium (100 mM CaCl₂, 6 mM NaTDC, 40 mM TriS-maleate, pH 6.5, 150 mM NaCl, 1000 TB units/ml lipase and colipase, 3.3% methyloleate, 37°C, crossed polars, first order quartz compensator).

Figure 5. The digestion of a 1,2-diolein droplet (D) by porcine pancreatic lipase and colipase after 17.5 minutes produces only liquid crystalline product phases (L) that undergo myelin figure formation (M) at pH 6.5 (8.3 mM CaCl₂, 83 mM NaTDC, 50 mM TriS-maleate, pH 6.5, 150 mM NaCl, 666 TB units/ml lipase, 1980 TB units/ml colipase, 5% 1,2-diolein, 23°C, Nomarsky, false colors).

Figure 6. The complete digestion of 1,2-diolein by porcine pancreatic lipase after 15 minutes at pH 8.3 in the absence of bile salts produces both crystalline (C) and isotropic liquid crystalline (L) product phases. The crystalline phase (C) appears secondarily in the isotropic phase. (1 mM CaCl₂, 100 mM Tris, pH 8.3, 150 mM NaCl, 930 TB units/ml lipase, 5% 1,2-diolein, 23°C, Nomarsky, false colors).

Figure 7. Product phases produced after 16 min. by lipase hydrolysis of 1,2-diolein. A different form of crystalline product phase (C) appears in a large pool of isotropic liquid crystalline product phase (L). (Conditions the same as Figure 6).

Figure 8. Digestion of 1,2-diolein by pancreatic lipase that shows a pool of liquid crystalline product phase (L) undergoing myelin figure formation (M). (after 20 minutes; same conditions as Figure 6).

Figure 9. Two Triglyceride droplets (T) after 15 minutes digestion by porcine pancreatic juice in the presence of porcine bile at pH 8.0. Note the tubular, myelin figure character (M) of some of the liquid crystalline phase (L) and small amounts of yellow crystalline phase (black arrow). (Total 25 µl mixture contained, 5 µl substrate mixture containing triolein: 10% gum arabic solution, 1:2 by volume, 40 mM Trits-maleate, 5 µl porcine gallbladder bile (approx. 200 mM bile salt partially saturated with lipid) and 15 µl fresh porcine pancreatic juice containing 403 TB units/ml lipase and colipase, 23°C, Normarsky).

Figure 10. A ring of transitory liquid crystalline product phase (H) shown colored here by Nomarsky optics appears during the hydrolysis of 1,2-diolein by pancreatic lipase and moves inward (black and white arrows) as the isotropic diolein (D) is digested. The isotropic product phase (L) also moves inward (black arrows) until (H) is completely transformed into (L). (after 21 minutes, same conditions as Figure 7).

Figure 11. The birefringent transitory product phase (H) shown in Figure 10 is transformed into isotropic liquid crystalline product phase. This figure shows sections of two rings as they blend (arrows) into the isotropic phase (L). Note the tubular projections (P) from the rings. (same conditions as Figure 7, phase contrast).

Figure 12. The appearance of pools of liquid crystalline product phase (L) in the intestinal contents (pH 7.1) of the killfish, Fundulus heteroclitus 1 hour after eating a fat meal. Droplets of undigested triglyceride (T) also appear mixed with the product phase. Intestinal tissue (I) occupies the upper right corner (frozen section post stained with Sudan Black B).

Figure 13. The partial digestion (after 15 minutes) of β-carotene saturated fat droplets (T) by human pancreatic lipase and colipase in the presence of human hepatic bile. Note how the β-carotene has moved into the isotropic liquid crystalline phase (L) (compare with Figure 3). The droplet to the right is still intact, the one in the center is completely digested and the one on the left is partially digested. (C) crystalline product phase (8 mM CaCl₂, 1.0 mM human bile salts, 0.3 mM lecithin, 40 mM TriS-maleate, pH 6.5, 150 mM NaCl, 3.3% triolein, and 1000 TB units/ml lipase and colipase, 37°C, bright field).

Figure 14. Liquid crystalline product phases (L) that have formed myelin figures (M) during the digestion of benzo(a)pyrene saturated (approx. 1.9%) fat droplets by porcine pancreatic lipase in the absence of bile salts, calcium and colipase. (After 20 minutes, 100 mM Tris, pH 8.3, 150 mM NaCl, 1500 TB units/ml porcine pancreatic lipase, 23°C, fluorescent illumination).

Figure 15. Section of fish intestinal villi 3 hours following feeding of benzo(a)pyrene saturated (1.9%) fat droplets. Lu – Lumen, V – villi. The meal consisted of a single piece of gelatin (35 mg) containing 20% triolein (+19% fluorescent benzo(a)pyrene). Note heavy fluorescence in tissue fat droplets (T). (Frozen section, fluorescent illumination).
of an individual fat droplet during lipolysis. Appearance to flow directly into the liquid crystalline product phases that form during fat digestion by pancreatic lipase in the absence of bile salts. Figure 14 shows a mass of fluorescent liquid crystalline product phase (L and M) that originates from fluorescent benzo(a)pyrene saturated (approx. 1.9%) long-chain triglyceride. There appears to be a hydrocarbon continuum or space in dietary fat that is preserved during lipolysis which allows molecules like the carcinogen benzo(a)pyrene to flow from oil phase to liquid crystalline phase (8,54). We have yet to see a fractionation of solute during digestion of long-chain triglyceride where part of the solute will pass into the product phases and part will remain in the droplet and finally crystallize as an insoluble remnant particle. However, when fluorescent benzo(a)pyrene was dissolved in medium-chain triglyceride (C8 acyl chains), a remnant crystal of the benzo(a)pyrene was left behind after the triglyceride was digested (not shown). This can be explained by the relatively high water solubility of octanoic acid (approx. 10^{-3} M compared to approx. 10^{-7} M for long-chain fatty acid) which did not form a liquid crystalline phase but instead dissolved in the water leaving behind the much more water insoluble benzo(a)pyrene (approx. 10^{-8} M) (2,44) to crystallize.

In the presence of saturated bile salt micelles, hydrophobic solutes also appear immediately in the liquid crystalline product phases. Figure 13 shows how β-carotene saturated (approx. 1%) fat droplets appear during digestion in the presence of lecithin saturated bile. Note the direct transfer of color from oil (T) to liquid crystalline phase (L). The dilution of color is caused by dilution of the product phase with water. When the bile salt micelles are not completely saturated, however, colored solutes may not appear in product phases. In Figure 3, the β-carotene saturated fat droplet (T) is clearly not transferring its color to the phases (L and C). It is difficult to tell whether or not the β-carotene has actually been excluded from the liquid crystalline phase (L) since the water content of this swollen phase may simply be so great that the color is diluted out. The crystalline phase is quite dense and not colored. Close examination of it, however, reveals small red crystals of β-carotene embedded in the otherwise uncolored phase (not visible in Figure 3). Thus some fractionation of solute can occur in the presence of unsaturated bile salt micelles.

Colored and fluorescent hydrophobic solutes can be used as probes to study lipid absorption. Figure 15 shows newly absorbed triglyceride (T) in the intestine of a fish that had been fed fluorescent benzo(a)pyrene saturated fat. The carcinogen and fat were codispersed, coabsorbed and coasimilized back into triglyceride droplets in the absorptive cells of the intestine (54). This classic experiment originally conducted with a red dye, was once thought to be proof that intact fat was absorbed until it was realized that dye and fat could have been absorbed individually and then recombined in the cell (37). Now that numerous fluorescent probes are commercially available, the molecular specificity of this coassimilation process may be determined.
The Light Microscopy of Triglyceride Digestion

Quantification of lipolysis by light microscopy

Theoretically light microscopy should be a simple method for quantifying lipolysis in very small samples. The rate of disappearance of the visible substrate should be proportional to enzyme activity. Unfortunately the crude 5–10 second mixing technique that we have employed in experiments to date does not uniformly mix substrate and enzyme. Some droplets or regions in the slide preparation are usually undergoing lipolysis at much greater rates than others. In some regions of the slide there may be a lag of 5–10 minutes before lipolysis begins. Often a single field of view in the microscope will exhibit droplets
that are completely digested beside others that remain unhidro-
ized. This problem is particularly noticeable in the absence of bile salts where pancreatic lipase irreversibly binds to sub-
strate in the first seconds of mixing. In the presence of bile salts the distribution of enzyme among substrate droplets is somewhat better although heterogeneity of mixing is still a problem. In addition, the particle sizes of our emulsions are also hetero-
geous. Once homogeneous emulsions and mixing are ob-
ained, it should be possible to accurately quantify lipolysis by measuring the change in light that passes through the slide pre-
paration as the fat droplets are digested.

Although we still have no accurate way of measuring lipase activity in a sample by microscopy, it is simple to measure the lipase activity that occurs on an individual fat droplet. The amorphous product phases with their variable water content are not easily quantified and in the presence of bile salts, product may not be visible at all. But the fat droplet itself, because it main-
tains its spherical shape during digestion, provides an accurate indicator of lipase activity. Figure 16a-f shows successive photos (30 sec. intervals) of the same field of emulsion droplets as they undergo lipolysis at pH 6.5 in the presence of bile salts. The particular region of the slide shown in Figure 15 did not begin to undergo lipolysis until 8 min. after the slide preparation was made. This lag may arise from diffusion of enzyme from an enzyme-rich area into an enzyme-poor area. Note the amorph-
ous liquid crystalline product phases (black and white arrows in Figure 16a). The change in diameters of five numbered fat droplets (shown in Figure 16) are plotted against time in Figure 17, which shows that the diameters of the fat droplets become smaller at an increasing rate. To the observer it appears that

the rate of digestion dramatically increases as the droplet dimin-
ishes in size. However, as Figure 18 shows the rate of change in volume of a fat droplet, which is the true measurement of
lipolysis, actually decreases with time. One possible explana-
tion for this result is that enzyme molecules are physically dis-
placed from the droplet surface as its surface area decreases (shown schematically in Figure 19). Also shown in figure 16 a-f are droplets which exhibited no change in dimensions with time (two examples have asterisks in Figure 16a). These may eventually be digested at a later time. It is not clear why.

The progress of lipolysis on a microscope slide can also be monitored by using radioactive substrates (40). The contents of the slide preparations can be rinsed with organic solvents into a test tube and the reaction stopped by acidification. The reaction components can then be separated by thin layer chromato-

graphy and quantified. Results with lingual lipase show that in the unstirred microscope preparation lipolysis is roughly linear for up to an hour (42).

**Conclusion**

The purpose of this brief review has been to illustrate how light microscopy can aid our understanding of fat digestion and the assimilation of hydrophobic solutes. It is undoubtedly clear to the reader that only the most preliminary experiments have been conducted and much additional experimentation is needed before some of the interpretations put forth in this review can be generally accepted. Although most of the phases that are shown are probably only transitory intermediates in the gastro-
intestinal tract, finding them has enabled us to better under-
stand how lipase functions, how lipids interact with ions and

water, and how hydrophobic vitamins and toxins are dispersed and assimilated in the intestine. By combining microscopy with biochemistry and biophysics the study of fat digestion can be eleva-
ted from a rather pedestrian subject to one of visual beauty. A color movie of many of the experiments discussed in this review is now available (43).

**Acknowledgements**

This work was supported by NIH grants AM 27304 and AM 18559 and a small grant from the Georgia Research Foundation to J.S.P. We thank Rhonda Tant, Michelle Taxel, and Mark Rigler for excellent technical assistance.

**References**

2. Bell GH. (1973). Solubilities of normal alipathic acids, alco-


*Large fat droplets (≥ 10 μm) may be flattened between the coverslip and slide and hence not spherical in shape. In addition, in the absence of support and sealant a cover slip will slowly fall as water rushes out and evaporates on the sides. This will flatten larger fat droplets and make them appear larger and dilute probe density.*
The Light Microscopy of Triglyceride Digestion


49. Stafford RJ, Donovan JM, Benedek GB, Carey MC. Physico-chemical characteristics of aqueous duodenal content after a fatty meal. Gastroenterol. 80:1291A.


Discussion with Reviewers

P. Berendsen: As "some dietary fat is partially crystallized at room temperature" did you use a warm stage to maintain the test slides at body temperatures during lipolysis and if so how did this influence the resulting products?

Authors: All of our experiments were conducted on liquid oils, most at room temperature. When we did use a warm stage we saw similar product phases, however the elevated temperature caused more rapid water evaporation and movement in unsealed slides. Although crystalline fats are slowly digested relative to liquid oils they should produce some very interesting new phases. We know nothing about such phases. It is conceivable that a liquid oil may produce only crystalline phases at a certain temperature. For example, trilinolein melts at –4°C to –5°C and oleic acid at 4°C. Lipolysis at 0°C may then produce solid products from a liquid substrate (even in the absence of calcium).

P. Berendsen: Would you speculate as to why some lipid droplets are lipolysed whereas others immediately adjacent are unaffected?

Authors: We add substrate and enzyme in separate solutions to the glass slide. Each addition is placed on the slide as a tiny droplet (5–20 μl). The reaction is then initiated by stirring the droplets together with the tip of a micropipette for several seconds. Lipase sticks rapidly to the nearest fat droplet and in the initial stage of the reaction most of the lipase will quickly associate with fat. Because of incomplete mixing fat droplets in some regions will acquire more enzyme than others. Mixing continues to occur, however, after most of the enzyme has already associated with nearby fat droplets so that droplets which were not near the initial set of enzyme may eventually lie next to droplets that received large doses of enzyme.

J.M. de Man: The paper deals with substances which are described as isotropic and anisotropic. How was this property established?

Authors: An isotropic (optically clear) phase was one that exhibited no birefringence by polarizing light microscopy (i.e., the phase was not visible). This phase was also optically clear when seen by Nomarsky interference optics.

J.M. de Man: How long would monoglycerides persist under conditions of pancreatic digestion?

Authors: Monoglycerides made up of a fatty acid esterified to the 1 or 3 position of the glycerol moiety would not be expected to persist at all since both pancreatic lipase and nonspecific lipase will attack primary fatty acid esters. However, pancreatic lipase does not attack 2-monoglycerides and in triglyceride digestions with pure pancreatic lipase at pH 7.0, 2-monoglycerides persist. The molar ratio of fatty acid to 2-monoglyceride after a 30 minute digestion with pure pancreatic lipase is roughly 2.

Acyl migration of the fatty acid from the 2 position to the 1 or 3 position of glycerol is a slow nonenzymatic isomerization that is accelerated at alkaline pH’s so in some pancreatic digests the 2-monoglycerides produced by lipase may undergo acyl migration to the 1 or 3 position and then be rapidly hydrolyzed by lipase. This is generally not a problem at neutral or acidic pH’s.

Pancreatic nonspecific lipase does attack 2-monoglycerides to a certain degree but its activity in pancreatic juice relative to pancreatic lipase appears to be low (text reference 41). The molar ratio of fatty acid to monoglyceride in intestinal content is always larger than 2, although this could be caused by more rapid absorption of monoglycerides relative to fatty acids (Ricour C and Rey J. 1970). Study of the oil and micellar phases during fat digestion in the normal child. Rev. Eur. Stud. Clin. Biol. 15:287–293, text reference 27). It is probably safe to say that a significant amount of 2-monoglyceride (i.e. > 10 percent) is hydrolyzed in vivo, primarily through the action of pancreatic nonspecific lipase.

A.F. Hofmann: Does an insoluble phase of calcium soap form and precipitate during fat digestion in man? What is the calcium activity of small intestinal content, and what determines it?

Authors: We do not know if calcium soap forms during digestion in man in vivo, however, with human pancreatic juice, human bile and human stomach content in vitro, we do see a calcium soap phase (text reference 41). We also do not know of any published values of calcium activity in small intestinal content. The following are published values of total calcium concentrations in human digestive fluids: total saliva, 1.5 mM; gastric juice, 0.15–1.23 mM; pancreatic juice (basal) – 0.26 mM; gall bladder bile, 3.7 – 10.8 mM; and intestinal juice (fasting) 4.2 mM (Lenter, C (Ed.).. Geigy Scientific Tables. Units of Measurement, Body Fluids and Nutrition, pp. I15-146, Ciba-Geigy Limited, Basel, Switzerland). Diet will clearly have profound effects on calcium concentration and activity. Dairy products are especially rich in calcium (see text reference 39).

A.F. Hofmann: Can you use a glycerol-2-mono-ether to explore the role of monoglyceride in preventing calcium precipitation with fatty acid?
The Light Microscopy of Triglyceride Digestion

Authors: That would be an excellent idea since the 2 mono­ether is much more resistant to hydrolysis than the ester. Its tendency to undergo acyl migration is probably also very small, although, we do not have data on that.

A.F. Hofmann: When you add buffer, it is “consumed” by titration of the fatty acid that is formed. Did you take this into account?

Authors: Yes it is consumed and yes we took this into account by calculating the maximum number of acid equivalents that our substrate could produce and then making sure that our buffer contained 3–4 times the capacity to neutralize that acid at the pH of the experiment. No matter how strong our buffer, we always saw a slight drop in pH (0.1 pH unit) during most digestions. Sodium bicarbonate is the buffer used in the intestine. To measure lipase activity in the pH stat we purposely make the buffer concentration very low so that lipase quickly over­rides it and lowers the pH.

A.F. Hofmann: If the lipase moves out with the product phase, how does it return to a new oil/water interface?

Authors: In vitro, where product dispersion is limited, the lipase molecule probably does not return to a new fat droplet. In vivo, where product is constantly being absorbed, product phases are continually being dispersed and lipase molecules are undoubtedly then freed to return to new oil/water interfaces.

K. Larsson: In “Results and Discussion,” it is stated that “the dominant liquid crystalline phase . . . appears as an amorphous isotropic pool”. This phase is said to have “a condensed rough lamellar structure.”

According to the reported optical data this phase must be the cubic phase. Do you think that the lamellar type of structure, which is proposed, is a texture effect only, that can be explained by deformation or flow of the sample?

Authors: We are not sure what a “texture effect” is but our freeze fractures of this isotropic phase, under a variety of conditions, always show a very condensed rough, layered phase (Rigler and Paton, in preparation). We have closely examined the extensive literature on the cubic phase and cannot reconcile our layered structure with cubic structure. We see lamellar structure within large isolated masses of product phase as well as adjacent to the surface of fat droplets. We doubt that the samples have been deformed or have flowed. Perhaps the real structure of the “cubic phase” has yet to be determined.

K. Larsson: The technique demonstrated in the paper in order to follow the fate of dissolved solutes is most interesting. Would it be possible to use different fluorescent amphiphiles, which are solubilized in the phases formed during digestion, in order to see whether or not lipid bilayers or product phases can fuse with the intestinal membrane?

Authors: Such experiments would be very difficult to interpret. We have used a variety of fluorescent lipid probes to examine the specificity of hydrophobic solute absorption (unpublished observations). In these experiments we simply dissolved the probe in dietary fat and looked for fluorescence in the reabsorbed fat (text reference 54). We have not taken lipid phases and intestinal tissue and tried any experiments of the kind you suggest. If one saw fluorescence in the cell it would be very difficult to determine the mechanism of cell entry and even if both lipid and probe were shown to enter simultaneously, one would not know if single monomers entered or if the product phases actually fused with the cell. By freeze fracture we do have evidence that lipolytic product phases can fuse with the intestinal membrane when the bile salt concentration is below the critical micellar concentration (Rigler and Paton, in preparation).