Watching Fat Digestion

The formation of visible product phases by pancreatic lipase is described.

John S. Patton and Martin C. Carey

Summary. During fat digestion a number of physicochemical events can be seen directly by light microscopy. Under simulated physiological conditions, hydrolysis of emulsified fat droplets by human pancreatic lipase in the presence of colipase and bile salt micelles proceeds with the sequential formation of two visible product phases. A lamellar liquid crystalline or crystalline phase containing calcium and ionized fatty acid forms first; this is followed by the production of a "viscous isotropic" phase composed predominantly of monoglycerides and protonated fatty acids.

The study of fat digestion began with a visual observation. In 1849, Claude Bernard observed that absorbed dietary triglyceride (fat) appeared only in those lymph vessels which originated distal to where the pancreatic duct enters the intestine of the rabbit (*1*). Since then our understanding of fat digestion has been derived almost entirely from measurements of chemical quantities and activities (*2*). Hofmann and Borgström in 1962 demonstrated the presence of an oil and presumed micellar phase in the intestinal content of man (*3*) and the process has repeatedly been viewed as a two-phase system (*4–6*). In this article we show that during fat digestion a sequence of physicochemical events occurs in which product phases are produced that are directly visible by light microscopy and are not readily dispersed by bile salts under physiological conditions. With both purified and natural components two distinct and visible product phases form sequentially from the oil phase in the presence of micellar bile sale solutions. Thus, fat digestion appears to consist of as many as four coexisting phases instead of two.

Fat digestion in most vertebrates occurs rapidly in the upper small intestine through the integrated action of pancreatic lipase, colipase, and bile. Colipase binds to the surface of fat droplets in the presence of bile salts and provides an attachment site for lipase (*7*). Lipase sequentially attacks the two outside ester bonds of the triglyceride molecule producing first a molecule of diglyceride and one of fatty acid and then two molecules of fatty acid and one of monoglyceride (*8*). Bile salts, which do not emulsify fats (*9*), are considered to incorporate these insoluble products of lipase hydrolysis into mixed micelles (*10*) from which fat absorption presumably occurs (*11*).

The components of fat digestion were combined under simulated physiological conditions (*5, 6, 12*) on a microscope slide and lipolysis (fat digestion) was observed by direct and polarized light microscopy to take place rapidly. Lipase and colipase were purified from human pancreatic juice as described (*13*), and in separate studies with natural components similar results were obtained (*14*).

The timed photographic sequence shown in Fig. 1, a to d, was taken during the same enzyme reaction. Intact olive oil droplets (*15*), after the addition of bile salts but before the addition of enzyme, are shown in Fig. 1a. Upon addition of lipase and colipase, the surface of the oil droplets became crenated within seconds as the first liquid crystalline product phase formed (Fig. 1b). As we watched the process the liquid crystalline shell became thicker until a critical stage was reached when the shell cracked and unhydrolyzed oil was expelled. Extrusion of oil droplets from the liquid crystalline product phase occurred within 3 minutes and by 3.5 minutes most particles had undergone phase separation (Fig. 1, c and d). In the absence of bile salts, fat droplets were transformed to the crenated stage but extrusion did not occur (not shown). The development of the birefringent liquid crystalline phase and the extrusion phenomenon also occurred in vitro in a natural system composed of stomach contents aspirated from a normal volunteer who had eaten a meal high in fat. Bile and pancreatic juice were added to the stomach contents to simulate conditions in the duodenum (Fig. 1e).

Figure 1f shows another example of an extruded oil droplet and its former liquid crystalline shell. After formation of the birefringent liquid crystalline product phase and extrusion, a second clear "viscous isotropic" phase emanated from the remaining oil droplets (*16–18*). Within 4 minutes (Fig. 1, g and h) an oil droplet diminished in size while the viscous isotropic phase increased. The final digestion sequence of a different particle (Fig. 1, i and j) shows the expansion of the viscous isotropic phase and a resulting remnant fat droplet. Remnant fat droplets persisted in the medium and never disappeared completely even in the presence of excess lipase. Unlike droplets of the oil phase which were always spherical, the viscous isotropic phase maintained no consistent shape, suggesting a very low interfacial tension, and sometimes formed birefringent myelin figures in the reduced water zones on the edge of the slide (*17–19*). Both product phases were partially soluble in unsaturated bile salt solutions under the relatively unstirred conditions of the slide experiments. When the bile salt concentration was low or saturated with lipids as with dilute natural bile (*20*), neither phase disappeared although digestion of the individual fat droplets to the remnant stage occurred. We observed the reappearance of small spherical fat droplets within the viscous isotropic phase as the slide preparation began to dry out; this is consistent with the reversibility of pancreatic lipolysis (*21*).

The phases observed microscopically were isolated from well-stirred bulk mixtures by centrifugation after 12-minute reaction times (Fig. 2). The lamellar liquid crystalline phase sedimented as a pellet even at low centrifugal forces, but not until it had separated from the oil phase by extrusion as observed microscopically. Its lipid composition in the purified systems was over 90 percent fatty acid (Fig. 2). Calcium-45 activity (15 mC/mg, 10 μC per experiment) was used to determine the calcium content of the

Dr. Patton is a postdoctoral fellow and Dr. Carey is assistant professor of medicine in the Department of Medicine, Gastroenterology Division, Harvard Medical School, Peter Bent Brigham Hospital, Boston, Massachusetts 02115.

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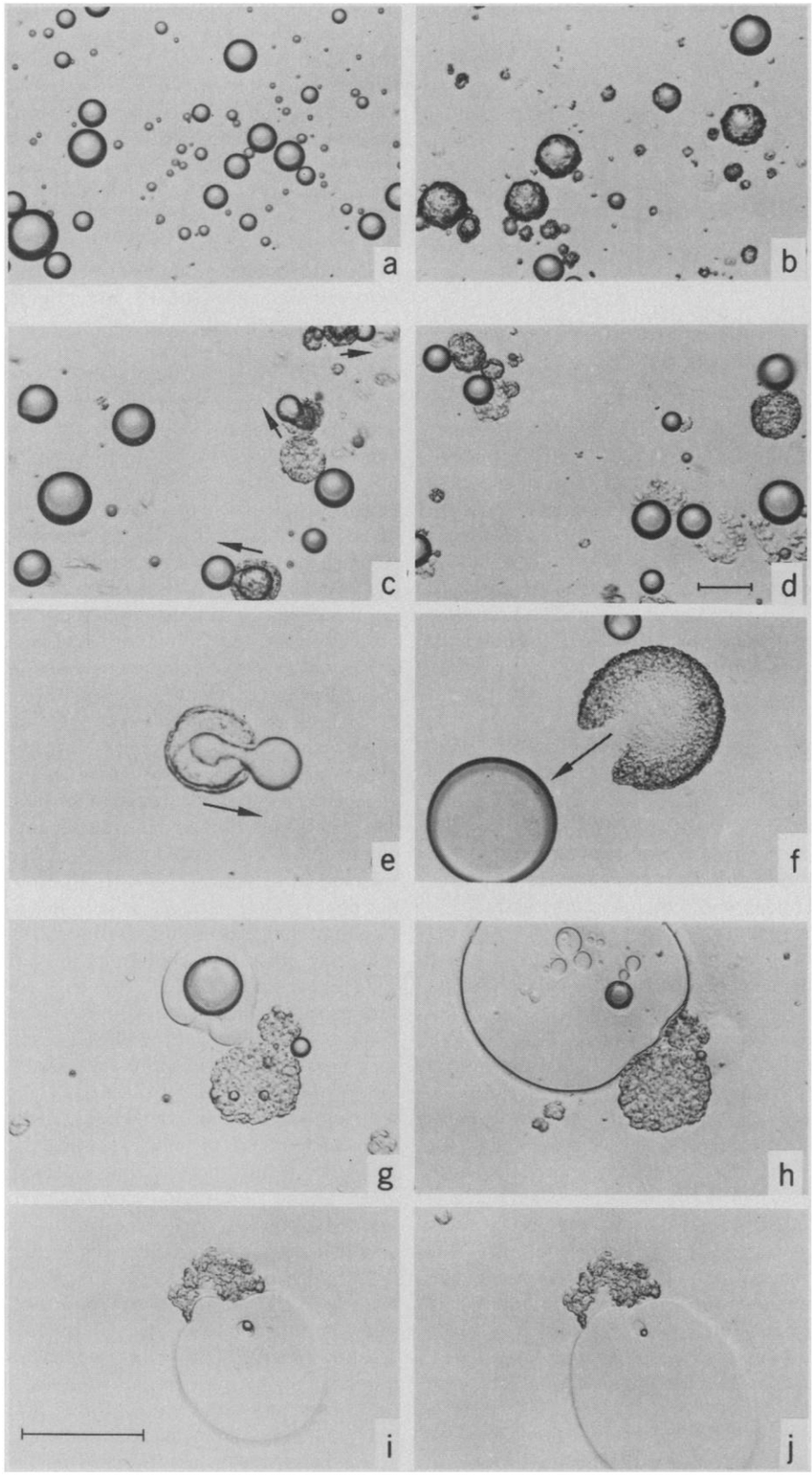

lamellar liquid crystalline phase. In three experiments the molar calcium to fatty acid ratio of the lamellar liquid crystalline phase was 1:2.1, which is identical to the theoretical value for calcium oleate "soap" formation (*22*). The floating oil phase recovered from the top of centrifuged mixtures contained mostly unhydrolyzed substrate (80 percent) as triglyceride and diglyceride (Fig. 2). The turbid supernatant contained mainly monoglycerides and fatty acids plus small amounts of diglyceride and triglyceride (Fig. 2) which, in reactions of longer duration (as in Fig. 3), reached product to bile salt molar ratios as high as 5. These results suggest that the viscous isotropic phase seen by light microscopy has a similar density to the micellar phase (*23*). The small amount of monoglyceride found in the pellet (Fig. 2) may result from the greater rate of solubilization of monoglyceride compared to protonated or calcium complexed fatty acids in bile salt solutions (*10*).

The appearance of product in the supernatant and pellet of a bulk-stirred reaction is shown plotted against time in Fig. 3. The amount of product incorporated into the pellet (calcium soap) closely parallels the amount of fatty acid ionized at *p*H 6.5. In similar experiments with low (1 m*M*) and high (10 m*M*) concentrations of calcium (Fig. 3, inset) the total hydrolysis was the same at both concentrations but the amount of product ionized was reduced with low calcium, and no pellet was formed. These findings are in agreement with the earlier observation that calcium promotes the ionization of fatty acids (*22*). Thus, with low calcium concentration at physiological *p*H, pancreatic lipase hydrolyzes fat and produces primarily protonated fatty acids. This suggests that the fatty acids of the calcium-containing phase are ionized while those of the viscous isotropic phase are protonated.

In a series of experiments with model systems, droplets of oleic acid containing increasing amounts of monoolein (5 to 50 percent by weight) were dispersed in buffer containing calcium (8 m*M*) and

Fig. 1. The sequence of events that occur during fat digestion on a microscope slide. Except for (e), all photomicrographs were taken of reactions containing purified components; the total volume was 30 μl and the final concentrations were 150 m*M* NaCl, 8 m*M* $CaCl_2$, 13 m*M* sodium taurodeoxycholate (bile salt), 40 m*M* tris maleate, *p*H 6.5, 3.3 percent purified olive oil, and 1000 tributyrin units per milliliter of human lipase and colipase (*13*). The photomicrographs (a to d) are taken from the same reaction sequence at 0, 1.5, 3.0, and 3.5 minutes, respectively. (a) Unhydrolyzed fat droplets; (b) formation of the first product phase (*22*) (lamellar liquid crystalline phase composed of calcium and fatty acid) (note: some droplets have escaped the initial "set" of lipase and colipase); (c) extrusion (arrows) of unhydrolyzed oil phase from the shell of lamellar liquid crystalline phase; (d) the separated oil and lamellar liquid crystalline phases; (e) extrusion as seen in the natural system of human bile (5 μl), pancreatic juice (15 μl), and postprandial stomach content (5 μl, reaction time 8 minutes); (f) a lamellar liquid crystalline shell and its extruded oil drop (reaction time 5 minutes). The sequences (g to h and i to j) are from separate reactions at approximately 20 to 28 minutes. Both sequences show the formation of a second nonbirefringent product phase, the "viscous isotropic" phase (*16*) composed of monoolein and protonated fatty acid. The small spherical droplet remaining within the viscous isotropic phase (j) is a fat droplet remnant. Scale bars, 100 μm.

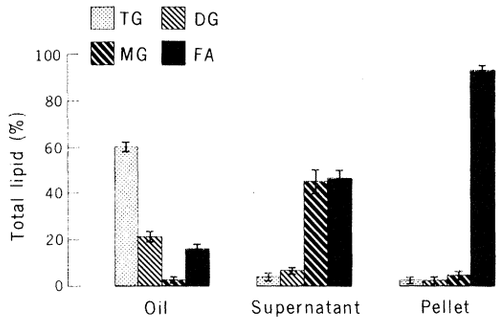

Fig. 2. The distribution of products and reactants of a lipase reaction after centrifugation at 4°C for 15 minutes at 50,000*g*. Reactions were run for 12 minutes in a *p*H-stat at *p*H 6.5 in a total volume of 10.0 ml containing final concentrations of 2 m*M* tris-maleate, 8 m*M* bile salt, 10 m*M* $CaCl_2$, 24 tributyrin units per milliliter of lipase and colipase, and 500 μl of an olive oil–gum arabic emulsion [olive oil and 10 percent gum arabic (1:2 by volume) emulsified by sonication] labeled with [1-^{14}C]-glyceryl trioleate (3×10^6 counts per minute; 41 millicuries per nanomole). Portions of the separated fractions were either counted for total radioactivity or were extracted, separated into lipid classes, and analyzed as previously described (*25*). The percentage of total lipids in the oil, supernatant, and pellet fractions were 82.8 ± 5.2, 13.1 ± 2.7, and 4.0 ± 1.7 percent, respectively. The pellet, which contained principally fatty acids and calcium, corresponds to the first product phase observed microscopically (lamellar liquid crystalline phase). The supernatant was a turbid two-phase system. This contained the second product phase observed microscopically, that is, a viscous isotropic phase (possibly a cubic liquid crystalline phase) in equilibrium with a saturated, mixed micellar phase comprised of lipid products and bile salts. The oil phase contained unhydrolyzed or partially hydrolyzed fat. Abbreviations: *TG*, triglyceride; *DG*, diglyceride; *MG*, monoglyceride; and *FA*, fatty acid. Vertical bars indicate 1 standard deviation of the mean of three experiments.

examined microscopically. In the absence of monoolein, oleic acid droplets formed birefringent calcium soaps. With small amounts of monoolein (5 percent), however, calcium soap formation was reduced and the resulting phase exhibited characteristics similar to the viscous isotropic phase. Thus monoglycerides, which accumulate during lypolysis, appear to determine the partitioning of lipid between calcium "soap" and viscous isotropic phases. For this reason addition of excess calcium to the final supernatant obtained in Fig. 3 produced no additional pellet upon centrifugation.

We have shown that two visible product phases occur during fat digestion. Both are presumably substrates which can be dispersed by bile salts to form a "mixed micellar" phase. The pellet from centrifuged intestinal content was analyzed only once previously (*24*) and was found to contain up to 15 percent of the total lipid. The second product phase, the viscous isotropic phase, has a density so similar to the micellar phase that the two cannot easily be separated. In previous attempts to isolate the phases of fat digestion from intestinal content, either ultracentrifugation or ultrafiltration was used (*3*–*6*). Prolonged ultracentrifugation is necessary to obtain a clear "micellar" phase; however, this treatment produces a marked bile salt–lipid gradient in the supernatant and only a small portion becomes clear (*5*). Our study suggests that a clear micellar phase in the supernatant may not occur during fat digestion because of the continuous production of the viscous isotropic phase, that is, the so-called micellar phase may always be a two-phase system. It is evident that the rate of formation relative to the rate of solubilization and absorption will determine the phase relationships occurring in vivo during fat digestion. Whether these liquid crystalline phases are substrates from which fat absorption occurs merits further investigation.

This article provides a new concept of fat digestion and demonstrates that the light microscope can be used to advantage in studying the action of an enzyme on an insoluble substrate, particularly when the products form liquid crystalline phases. In addition it suggests a simple microtechnique for detecting the activity of any lipase on its substrate and for following the sequence cinematographically.

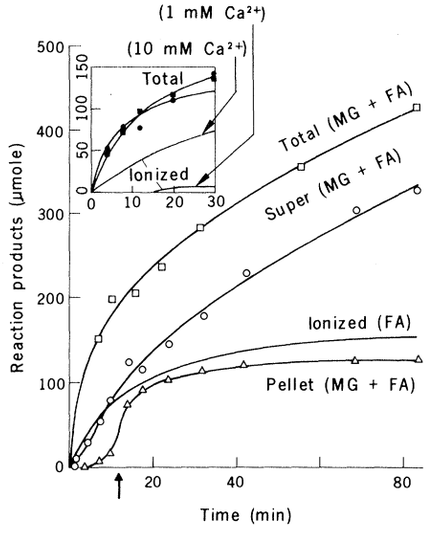

Fig. 3. Analysis of the products formed from a single lipase reaction as a function of time. Reaction conditions were the same as in Fig. 2 except the purified lipase, colipase, and olive oil concentrations were doubled. At various time points from the initiation of the reaction, 200-μl samples of the stirred reaction mixture were withdrawn by micropipette. Some samples were extracted whole to obtain total product formation (□); other samples (200 μl) were first fractionated in a microcentrifuge into supernatant (*Super*) (○) and pellet (△) fractions which were then individually analyzed to give micromoles of product. The extent of ionization of the fatty acids [*Ionized (FA)*] produced in the reaction was measured continuously with a *p*H-stat (0.1*N* NaOH, *p*H 6.5). The arrow beneath the abscissa indicates the approximate time at which extrusion of oil droplets from the first product phase occurred microscopically. (Inset) Conditions were the same as in Fig. 2, except that total product formation was assayed in the presence of 10 m*M* (■) or 1 m*M* (●) $CaCl_2$; the curves marked *Ionized* indicate the degree of ionization of fatty acids. Abbreviations as in Fig. 2.

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14. The pancreatic juice contained 600 tributyrin units per milliliter of lipase and colipase; the hepatic bile (total fistula patient) contained 6 m*M* total bile salt and 1.6 m*M* lecithin; and the stomach content contained a meal of barbecued potato chips (source of fat droplets) which had a residence time of 1.5 hours in the stomach of a normal volunteer.
15. Sonicated or hand-shaken gum arabic emulsions provided the most stable preparations (10 percent gum arabic and olive oil, 2:1 by volume). Similar results were obtained with sodium oleate–stabilized emulsions (1 percent oleate), but these emulsions were less stable at *p*H 6.5. Solutions of gum arabic (Sigma) were first dialyzed to remove divalent salts [W. J. Brown, A. A. Belmonte, P. Melius, *Biochim. Biophys. Acta* **485**, 313 (1977)].
16. To avoid committing ourselves to structure type, this second nonbirefringent phase is here called simply "viscous isotropic" to distinguish it from the first birefringent phase which exhibited optical textures of a neat soap or "acid-soap" (*22*) of smectic (possibly lamellar) type. Two liquid crystalline phases have been identified in pure aqueous-monoolein systems at ambient temperatures by polarizing microscopy and x-ray diffraction [Lutton (*17*); K. Larsson, *Z. Phys. Chem. N. F.* **56** (1967)]. Between 4 and 22 percent H_2O a lamellar phase, and between 25 and 41 percent H_2O a "viscous isotropic" phase occurs. At higher H_2O contents the latter phase coexists with excess water. Both freeze-etch electron microscopy [Lundberg (*18*)] and x-ray diffraction [K. Larsson, *Chem. Phys. Lipids* **9**, 181 (1972)] suggest that the viscous isotropic phase of the monoolein-water system consists of spherical water droplets in a cubic face centered lattice surrounded by a hydrocarbon continuum. The retention of the optical viscous isotropic appearance of this phase with incorporated fatty acids in a bile salt solution suggests a similar molecular structure for the viscous isotropic phase during fat digestion.
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23. In reactions with natural bile, where the final bile salt concentration was low (2 m*M*) and the micelles saturated with lecithin (*20*), the viscous isotropic phase floated with the oil phase; this may explain the high product content of the oil phase observed by Hofmann and Brogström (*3*) and others who used ultracentrifugation techniques (*4–6*).
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A Two-Fluid Approach to Town Traffic

Robert Herman and Ilya Prigogine

A kinetic theory of vehicular traffic has been developed over the past 20 years to attempt to describe the characteristics of traffic on multilane highways (*1–3*). In this theory we have examined the evolution of the speed distribution function in terms of a number of important processes: the relaxation or speeding up process, which expresses the attempts of drivers to achieve their own desired speeds; the interaction or slowing down process, which arises in the conflict between a faster driver and a slower driver; and the adjustment process, which reduces the variance around the local mean speed. We have tried to avoid examining many specific details—for example, the details of all the vehicles in queues and the details of passing maneuvers. In addition, since the beginning of this work, we have been searching for an extension of the theory that would describe in a similar overall fashion the traffic in towns—that is, traffic in an extended network.

Traffic Observations

In order to obtain some feeling for such a complex problem we have made a number of observations. Recently, we analyzed a large amount of data in the form of speed-time histories of vehicles reported by Johnson *et al.* (*4*) for many cities in the United States. The data were generated by following vehicles in each area studied, and they consist of speed-time histories and usage patterns of randomly selected vehicles operated under different traffic conditions as well as on various roadways. Using these data, we have investigated the possibility of evaluating relative traffic quality and finding ways to characterize traffic on different roadway types with various flow levels in nine metropolitan areas (*5*). We have examined traffic attributes such as average speed, stopped time, speed distribution function, and acceleration and speed noise (defined as the standard deviation of acceleration or speed), as well as the ratio of speed noise to average speed (the coefficient of variation of speed), in order to measure quantitatively the effects of road type, traffic conditions, and driver behavior on traffic phenomena.

We have found that although the traffic in various areas is different, similar relations appear to exist between a num-

Robert Herman is a research fellow at General Motors Research Laboratories, Warren, Michigan 48090. Ilya Prigogine is director of the Center for Statistical Mechanics and Thermodynamics, University of Texas, Austin 78712, and head of the Chemical Physics Department at the Université Libre de Bruxelles, Brussels, Belgium.

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