

Retention of lipolytic products in chylomicrons incubated with lipoprotein lipase: electron microscope study

E. Joan Blanchette-Mackie and Robert O. Scow

Section on Endocrinology, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Abstract Early effects of lipolysis on the structure of chylomicrons in vitro were studied in rat chylomicrons incubated with purified bovine milk lipoprotein lipase at pH 8.1. The amount of the albumin added to the incubation medium was limited so that free fatty acids (FFA) and partial glycerides formed during lipolysis would accumulate in the chylomicrons. The structures visualized in lipolyzed chylomicrons was found to be affected by pH during preparation of specimens for microscopy, whether fixed with OsO₄ and sectioned, or stained with sodium phosphotungstate and examined as whole mounts. Circular aqueous spaces were present in the triglyceride core of lipolyzed chylomicrons processed at pH 8.1 and 7.4. Sometimes the spaces contained aggregates of osmiophilic material and whorls of bilayered lamellae. The spaces were replaced by lamellar structures, having a periodicity of 40 Å, in chylomicrons processed at pH 5.5, and the spaces and lamellae were both absent at pH 3.0. The findings indicate that these spaces were lined by a lipid monolayer which formed bilayered lamellae under certain conditions. It is concluded that the monolayer lining the aqueous spaces is an inward extension of the chylomicron surface film produced by the accumulation and movement of lipolytic products, FFA and partial glycerides, in the interfacial plane between core triglyceride and water.

Supplementary key words triglyceride • diglyceride • monoglyceride • free fatty acids • bilayered lamellae • lipid monolayer • osmium tetroxide • phosphotungstic acid

Recent studies of chylomicrons incubated with lipoprotein lipase showed that chylomicrons are enveloped by a monolayer surface film (1). The film was visualized in sections of lipolyzed chylomicrons as a thin electron-opaque line (25–30 Å wide) in areas where the underlying triglyceride core was replaced by zones of decreased electron opacity, and as a fold of surface film extending outward from the chylomicron core. The findings also showed that chylomicrons assumed many different shapes as they were being depleted of triglyceride and that free fatty acids (FFA) accumulated in lipolyzed chylomicrons when fatty acid binding sites on albumin were not available. Lipoprotein lipase for those studies was obtained by adding chylomicrons to postheparin plasma and isolating the substrate-enzyme complex by several centrifugations. Unfortunately, considerable hydrolysis occurred during

isolation of the complex, and the amount of enzyme activity obtained could not be controlled.

The present investigation was carried out using lipoprotein lipase purified from bovine milk (2–4) to determine the immediate structural transformations induced in chylomicrons by lipoprotein lipase in vitro. The effect of various preparatory techniques (fixation and staining) on the appearance of lipolyzed chylomicrons was also studied.

METHODS

Preparation of chylomicrons

Doubly labeled chylomicrons were isolated from thoracic duct chyle collected for 6 hr from starved rats after they were tube-fed 0.5 ml corn oil containing tri-(1-¹⁴C)oleoylglycerol (15 mCi/mmol, Lot 573663, Chemical and Radioisotopes Division, International Chemical and Nuclear Corp., Irvine, Calif.) and trioleoyl-[2-³H]glycerol (2 mCi/mmol, Lot No. 733864, International Chemical and Nuclear Corp.) (5). The rats used were adult Charles River females (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) fed ad libitum Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.). The chyle was centrifuged in a swinging head rotor (SW-39 or SW-50) at 24,000 rpm for 60 min at 3°C with a Spinco model L or L-2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The compact floating layer of chylomicrons formed during centrifugation was collected and suspended, as described elsewhere (5), in 0.6 mM albumin-Tyrode's solution [bovine serum albumin powder (Fraction V, Armour Pharmaceutical Co., Chicago, Ill., Lot G-36009), 40 mg/ml, in a glucose-free Tyrode's buffer solution], pH 7.4, at a triglyceride concentration of 70–110 mM. The suspension was stored at 4°C and used for experiment within 7 days.

Source of lipoprotein lipase

The preparation of lipoprotein lipase used in these studies

Abbreviations: BS/FA, binding sites on albumin per molecule of fatty acid; FFA, free fatty acid.

was highly purified from bovine skim milk (2) and was kindly supplied by Drs. Torbjorn Egelrud and Thomas Olivecrona of the University of Umeå, Sweden. The solution of enzyme used contained 170 μg of protein/ml and had 83 units of lipolytic activity/ml (1 unit = μmol FFA released/min at 24°C) (3). The enzyme was stored in liquid nitrogen and lost practically no activity during storage for 8 months. Small aliquots of the frozen enzyme solution were thawed for use immediately prior to each experiment.

Incubation of chylomicrons

Chylomicrons suspended in albumin-Tyrode's solution were mixed with lipoprotein lipase at room temperature for 15–30 sec, and then added to 0.6 mM albumin solution buffered with either 0.1 M Tris (tris[hydroxymethyl]amino-methane)-HCl or 0.1 M sodium veronal-HCl, pH 8.1, for immediate incubation at 38°C. Usually 0.4–0.5 unit of enzyme activity was added per μmole of chylomicron triglyceride and the concentration of chylomicron triglyceride in the incubation mixture was 8–13 mM. Preliminary studies had shown that chylomicrons incubated with lipoprotein lipase released no more than 6 molecules of FFA per molecule of albumin in the incubation medium. Consequently, the number of binding sites on albumin available per molecule of fatty acid in the incubation mixture, BS/FA, ranged in most experiments from 0.09 to 0.15 (BS/FA = from mM albumin \times 6) / mM triglyceride \times 3). Aliquots of the incubation mixture were taken at various times for morphological and biochemical studies. Sometimes, chylomicrons were separated from the incubation mixture by ultracentrifugation in a swinging head rotor at 24,000 rpm for 60 min at 3°C and resuspended in various solutions for morphological and biochemical studies.

Chylomicrons markedly depleted of triglyceride (triglyceride-depleted chylomicrons) were prepared as follows: chylomicrons were incubated with lipoprotein lipase for 30–60 min in medium (1.2 mM albumin–0.1 M Tris solution, pH 8.1) containing sufficient albumin to bind 100–140% of the fatty acids (BS/FA = 1.0–1.4). The incubation mixture was then centrifuged in a swinging head bucket at 24,000 rpm for 60 min at 3°C and the resultant clear infranatant was collected and mixed with sufficient KBr (0.37 g/ml incubation mixture) to increase the density to 1.23 g/ml. The latter was then centrifuged either in a high force fixed angle rotor (Type 65, Beckman Instruments, Inc., Palo Alto, Calif.) at 65,000 rpm for 18 hr at 4°C or in a swinging head rotor (SW-50.1) at 49,000 rpm for 23 hr at 0°C, and the resultant floating film was collected for morphological and biochemical studies.

Biochemical analyses

Lipids in chylomicrons and incubation mixtures were extracted into hexane (6) and separated into triglyceride, diglyceride, monoglyceride and FFA fractions by thin-layer chromatography (7). The lipids were dissolved in 15 ml of toluene containing 4.2% Liquifluor (New England Nuclear Corp., Pilot Chemicals, Inc., Boston, Mass., cat. no. NEF-903) for measurement of ^3H and ^{14}C content with a liquid scintillation spectrometer (Packard Tri-Carb Model 314-EX,

TABLE 1. Effect of pH during preparation of specimens on structure visualized in the core of lipolyzed chylomicrons

pH during preparation ^a	Structure visualized	
	In electron-opaque core of chylomicrons fixed with OsO_4 and sectioned	In electron-lucent core of chylomicrons stained with sodium phosphotungstate
8.1	Many electron-lucent circular areas	Many electron-opaque circular areas
7.4	Many electron-lucent circular areas	Many electron-opaque circular areas
6.0	Few electron-lucent areas	Few electron-opaque areas
5.5	Many lamellae	Many lamellae
5.3	Many lamellae	Many lamellae
3.0	None	None

^a The solutions used for suspending lipolyzed chylomicrons after centrifugation and for fixing specimens with OsO_4 were buffered with 0.1 M sodium cacodylate-HCl at pHs 5.3–8.1 and with 0.1 M sodium citrate-HCl at pH 3.0. The sodium phosphotungstate solutions used for staining were prepared at various pHs by adding different amounts of NaOH to 2% phosphotungstic acid in water.

Packard Instrument Co., Inc., Downers Grove, Ill.) (1). The gain and discriminators were set so that ^3H was counted with an arbitrary efficiency of 100% in channel A and less than 1% in channel B, and ^{14}C was counted with an arbitrary efficiency of 100% in channel B and 28–30% in channel A. The amount of chylomicron triglyceride hydrolyzed to glycerol and FFA was measured by decrease in the ratio of ^3H to ^{14}C in the hexane extract of the incubation mixture (8). The triglyceride content of chylomicrons was estimated by the method of Rapport and Alonzo (9).

Morphological analyses

Osmium tetroxide-fixed specimens. Chylomicron samples were fixed overnight in cold buffered 2% OsO_4 solution at different pHs ranging from 8.1 to 3.0 (see Table 1) and then centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div. Palo Alto, Cal.) at 12,000 rpm for 30–60 sec. The resultant pellet containing fixed chylomicrons was dehydrated rapidly with acetone and embedded in Epon (10). Thin sections of the embedded samples were stained with lead hydroxide and examined in a Philips EM 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N.Y.).

Sodium oleate was prepared by adding 4 μg of oleic acid to 0.2 ml of 0.1 M sodium cacodylate solution at pH 7.4. The flaky precipitate formed after several minutes was fixed overnight in 2 ml of cold 2% OsO_4 in 0.1 M sodium cacodylate solution at pH 7.4, collected by centrifugation, and processed as above for electron microscopy.

Sodium phosphotungstate-stained specimens. Chylomicron samples were spread directly on grids coated with Formvar, stained for 1 min with 2% sodium phosphotungstate solution at various pHs ranging from 8.1 to 3.0 (see Table 1), rinsed

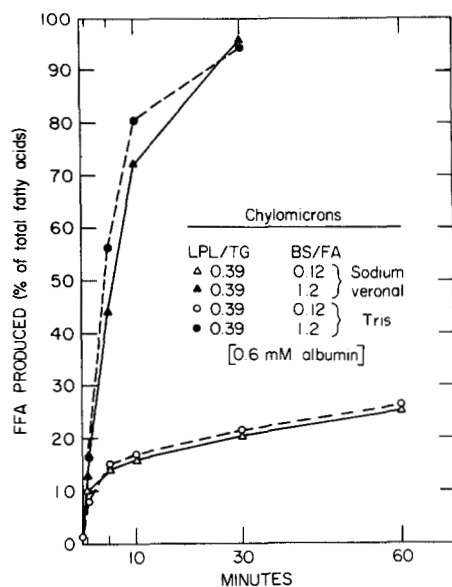


Fig. 1. Effect of availability of fatty acid binding sites (BS/FA) and kind of buffer in the incubation medium on hydrolysis of chylomicron triglyceride by lipoprotein lipase. The incubation medium contained 0.6 mM albumin and 0.1 M Tris or 0.1 M sodium veronal at pH 8.1. LPL/TG = units of lipoprotein lipase activity per μmol of chylomicron triglyceride, and BS/FA = number of fatty acid binding sites on albumin (6/molecule) available per molecule of fatty acid in incubation mixture. BS/FA was increased by decreasing the amount of chylomicrons added to the incubation mixture. The values given are means of two experiments.

TABLE 2. Effect of albumin and buffer on the hydrolysis of chylomicron triglyceride by lipoprotein lipase

Duration of incubation <i>min</i>	Initial ratio of triglyceride to albumin		Buffer	Incubation mixture				$[^{14}\text{C}]$ oleic acid present as FFA
	<i>mM/mM</i>	BS/FA		Distribution of $[^3\text{H}]$ glycerol				
				Triglyceride	Diglyceride	Monoglyceride	Glycerol	
0						% of total		
10	17.	0.12	Tris	95.4 \pm 0.1	3.4 \pm 0.1	0.7 \pm 0.1	0	1.5 \pm 0.1
10	17.	0.12	Sodium veronal	76.0 \pm 0.5	11.0 \pm 0.5	7.0 \pm 0.4	5.0 \pm 0.1	17.0 \pm 0.3
60	17.	0.12	Tris	76.0 \pm 0.5	11.5 \pm 0.1	7.5 \pm 0.1	4.5 \pm 0.5	17.0 \pm 0.5
60	17.	0.12	Sodium veronal	63.5 \pm 0.2	14.0 \pm 0.1	9.0 \pm 0.2	14.0 \pm 0.1	26.2 \pm 0.2
30	1.7	1.2	Tris	64.0 \pm 2.0	13.0 \pm 0.5	9.5 \pm 0.1	12.5 \pm 0.8	26.2 \pm 0.7
30	1.7	1.2	Sodium veronal	2.7 \pm 0.9	0.2 \pm 0.1	8.1 \pm 1.5	87.0 \pm 1.0	94.6 \pm 0.1
				3.5 \pm 0.5	0.5 \pm 0.1	5.5 \pm 1.0	91.0 \pm 1.0	96.0 \pm 0.1

Chylomicrons containing glyceride labeled with $[^3\text{H}]$ glycerol and $[^{14}\text{C}]$ oleic acid were incubated at 38°C with lipoprotein lipase. 0.39 unit/ μmol of triglyceride in different amounts of 0.6 mM albumin solution buffered with either 0.1 M Tris or 0.1 M sodium veronal (pH 8.1). BS/FA = total number of fatty acid binding sites on albumin available per molecule of fatty acid in the medium = (mM albumin \times 6)/(mM triglyceride \times 3). Values given are means \pm SE of two experiments.

TABLE 3. Distribution of $[^{14}\text{C}]$ oleic acid after incubation of chylomicrons with lipoprotein lipase in medium containing limited albumin (BS/FA = 0.14)

Duration of incubation <i>min</i>	In chylomicron				In medium			
	Triglyceride	Diglyceride	Monoglyceride	FFA	Triglyceride	Diglyceride	Monoglyceride	FFA
0	95.8 \pm 0.7	1.9 \pm 0.2	0.6 \pm 0.2	1.6 \pm 0.5	0	0	0	0
5	71.8 \pm 2.5	2.8 \pm 0.2	2.2 \pm 0.1	5.0 \pm 0.1	0.8 \pm 0.1	0.4 \pm 0.2	0.7 \pm 0.4	16.6 \pm 2.4

Chylomicrons containing glyceride labeled with $[^{14}\text{C}]$ oleic acid were incubated with lipoprotein lipase (LPL/TG = 0.51) at 38°C in 0.6 mM albumin - 0.1 M Tris solution (pH 8.1) and separated from the medium by ultracentrifugation. Values given are means \pm SE of three experiments.

with water, air-dried, and viewed directly, as whole mounts, with the electron microscope.

RESULTS

Biochemical studies

Triglyceride in chylomicrons was readily hydrolyzed by lipoprotein lipase to FFA and glycerol when incubated in a medium containing sufficient albumin to bind all of the FFA produced (BS/FA = 1.2), (Fig. 1 and Table 2). However, if albumin was limited (BS/FA = 0.12), lipolysis slowed down as soon as FFA production exceeded the capacity of albumin to bind fatty acids (mole of FFA produced/mole of total fatty acids $>$ 0.12) (Fig. 1), and FFA accumulated with monoglyceride and diglyceride in the chylomicrons (Table 3). Changing the buffer of the incubation medium from Tris to sodium veronal had no effect on the rate of hydrolysis (Fig. 1 and Table 2), but it did facilitate visualization of lamellae in lipolyzed chylomicrons prepared at pH 5.5 (see below).

Morphological studies

Effects of lipolysis on structure were studied in chylomicrons incubated 4–10 min with lipoprotein lipase at pH 8.1 in a medium containing only enough albumin to bind 9–15% of the fatty acids present in the incubation mixture (BS/FA = 0.09–0.15). Changes in structure were visualized in sections after OsO_4 fixation and in whole mounts after staining

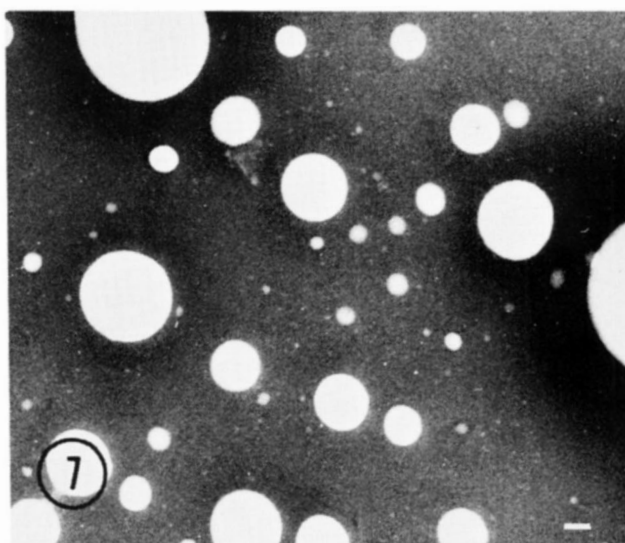
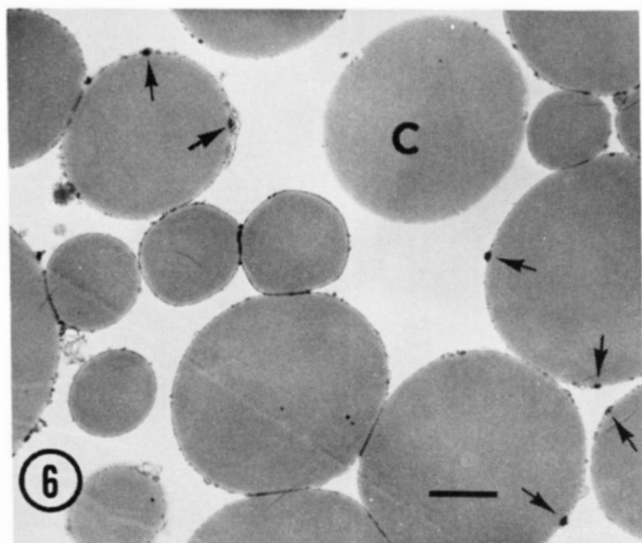
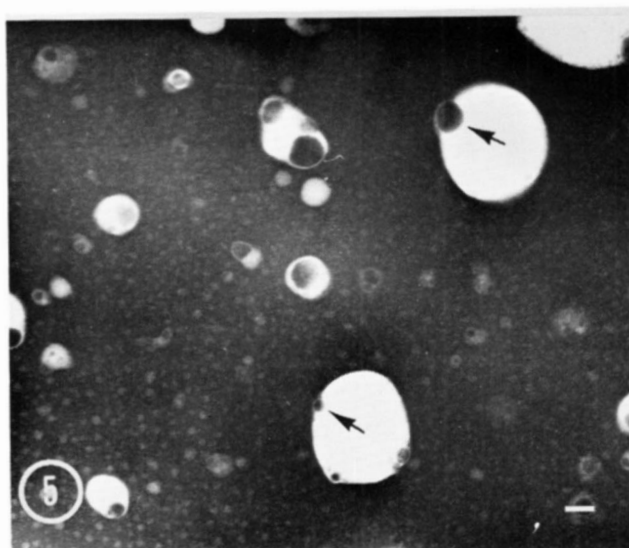
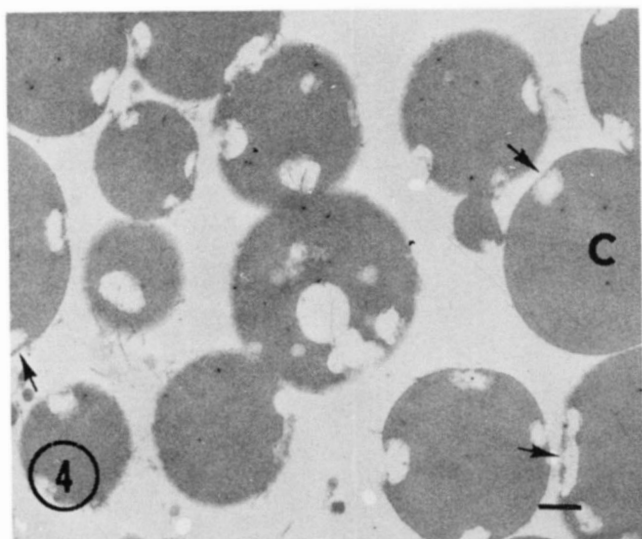
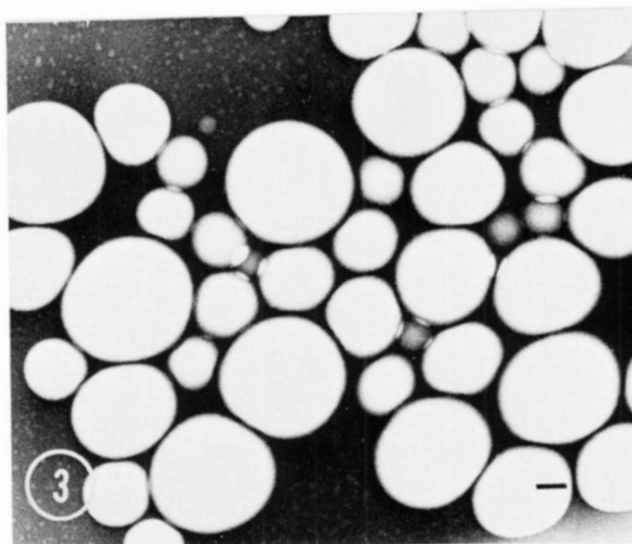
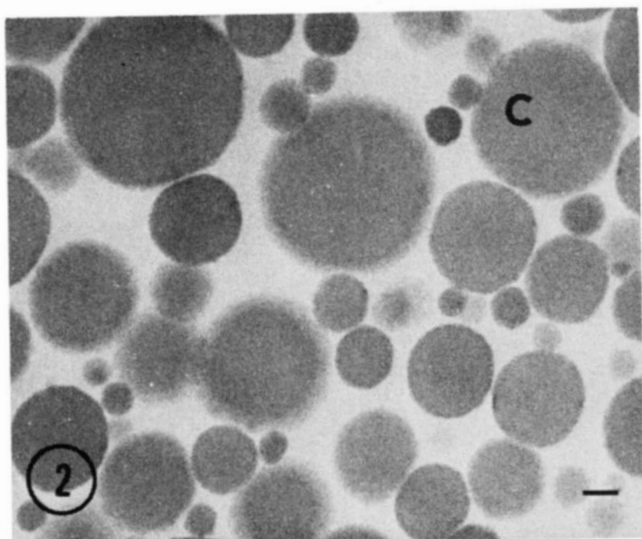


Fig. 2. Control chylomicrons were fixed with OsO_4 at pH 8.1, centrifuged and sectioned. The chylomicron core (C) is smooth, circular and uniformly electron-opaque. The indistinct periphery of some of the chylomicrons is due to oblique sectioning of the surfaces. The marker represents $0.1 \mu\text{m}$ on all figures. $\times 50,000$.

Fig. 3. Control chylomicrons were stained with sodium phosphotungstate at pH 7.4 and examined as whole mounts. The chylomicrons appear as electron-lucent spheres. $\times 40,000$.

Fig. 4. Chylomicrons were incubated with lipoprotein lipase for 7 min in Tris-buffered medium, fixed with OsO_4 at pH 7.4, centrifuged and sectioned. Electron-lucent circular areas are present within the chylomicron core (C). Note the narrow strands, similar in electron-opacity to the core, demarcating electron-lucent areas at the periphery of chylomicrons (arrows). BS/FA = 0.11; FFA/TFA = μmol FFA/ μmol total fatty acids in incubation mixture = 0.08. $\times 60,000$.

Fig. 5. Chylomicrons were incubated with lipoprotein lipase for 5 min in Tris-buffered medium, stained with sodium phosphotungstate at pH 7.4 and examined as whole mounts. Numerous electron-opaque circular areas (arrows) of various sizes are present, at the periphery, in the chylomicrons. Note that many of the chylomicrons bulge in the region of the electron-opaque areas. The electron-opaque areas, which are stained with sodium phosphotungstate, indicate that aqueous spaces are present in the chylomicrons, and that these spaces communicate directly with the surrounding aqueous medium. BS/FA = 0.11; FFA/TFA = 0.08. $\times 35,000$.

Fig. 6. Chylomicrons were incubated with lipoprotein lipase for 10 min in medium buffered with sodium veronal, separated from the incubation medium by ultracentrifugation, suspended in 0.1 M sodium cacodylate solution (albumin-free) at pH 5.5, fixed in OsO_4 at pH 5.5, centrifuged and sectioned. These chylomicrons do not have electron-lucent areas, as in Fig. 4, but instead have numerous osmiophilic deposits at the periphery (arrows). BS/FA = 0.15; FFA/TFA = 0.17. $\times 90,000$.

Fig. 7. Chylomicrons were incubated with lipoprotein lipase for 4 min with Tris-buffered medium, separated from the incubation medium by ultracentrifugation, suspended in buffered 4% albumin solution (pH 5.5), stained with sodium phosphotungstate at pH 5.5 and examined as whole mounts. FFA accounted for 8.1% of the fatty acids present in the chylomicrons. Note that these chylomicrons appear as regular electron-lucent spheres, not unlike control chylomicrons (Fig. 3), and that very few of them have electron-opaque areas (compare with Fig. 5). (This micrograph also shows that lipolyzed chylomicrons maintain their integrity even after being mixed with sodium phosphotungstate at pH 5.5) BS/FA = 0.12; FFA/TFA = 0.20. $\times 32,000$.

with sodium phosphotungstate. Since pH of the solutions used for preparation of specimens was found to influence the structure seen in lipolyzed chylomicrons, a systematic study was made of the effect of pH on structure. Therefore, control and lipolyzed chylomicrons were fixed with OsO_4 or stained with sodium phosphotungstate at various pHs ranging from 8.1 to 3.0 (Table 1).

Control chylomicrons: pH 8.1–3.0. Chylomicrons isolated from thoracic duct lymph, fixed in OsO_4 at pH 8.1, and sectioned are shown in Fig. 2. The core of the chylomicrons was circular and uniformly electron-opaque, and the periphery was free of granules. Chylomicrons fixed at pH 7.4, 6.0, 5.5 and 3.0 were similar in appearance.

Control chylomicrons stained with sodium phosphotungstate at pH 7.4 appeared as electron-lucent spheres (Fig. 3). Chylomicrons stained at pH 8.1, 6.0, 5.5 and 3.0 also appeared as spheres.

Lipolyzed chylomicrons: pH 8.1. Lipolyzed chylomicrons fixed with OsO_4 at pH 8.1 contained numerous circular electron-lucent areas, often located near the periphery of the electron-opaque core (Fig. 8a). Some of the electron-lucent areas contained small electron-opaque granules. Lipolyzed chylomicrons stained with sodium phosphotungstate at pH 8.1 contained many electron-opaque spaces near the periphery of the electron-lucent lipid core (Fig. 8b). Since these structures resembled those seen in specimens prepared at pH 7.4, the pH routinely used for tissue preparation, they are described below in more detail.

Lipolyzed chylomicrons: pH 7.4. The structure of lipolyzed chylomicrons fixed with OsO_4 at pH 7.4 was similar to that

observed in chylomicrons incubated with lipoprotein lipase obtained from postheparin plasma (1). Numerous circular electron-lucent areas were present in the homogeneously electron-opaque core (Figs. 4, 8c and 9a). The electron-lucent areas near the periphery were usually separated from the exterior by narrow strands similar in electron opacity to that of the core (Figs. 4, 8c and 9a). In some sections, a bilayered lamellae, 40 \AA in width, separated the electron-lucent areas from the outside (Figs. 9a and b). The outer layer of the lamella was continuous with the chylomicron surface film, and the inner layer continuous with the monolayer lining the electron-lucent area (Fig. 9b). Aggregates of osmiophilic material (Fig. 8a) and circular configurations of bilayered lamellae (Figs. 9a and 9b) were seen in some of the electron-lucent areas.

Lipolyzed chylomicrons stained with sodium phosphotungstate at pH 7.4 contained numerous electron-opaque areas in the electron-lucent core (Figs. 5 and 8d). Although the areas were usually small and located near the periphery, they sometimes occupied a large part of the chylomicron core (Fig. 5). The electron-opaque areas were separated from each other by thin electron-lucent walls (Fig. 5). Although the chylomicrons were usually spherical, they sometimes bulged outward in the vicinity of the electron-opaque areas (Figs. 5 and 8d). The electron-opaque areas in chylomicrons stained with sodium phosphotungstate are probably accumulations of the stain in spaces which communicated with the surrounding aqueous medium. It is likely that these spaces correspond to the electron-lucent areas seen in sections of chylomicrons fixed with OsO_4 .

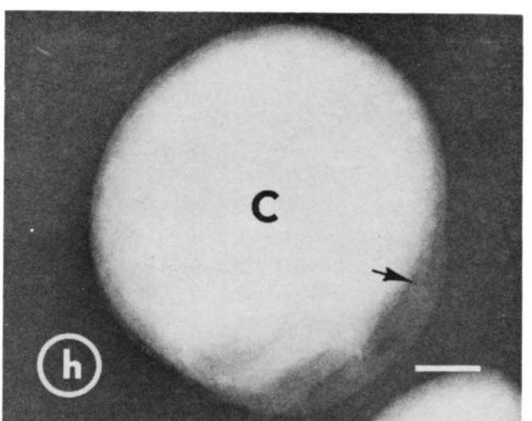
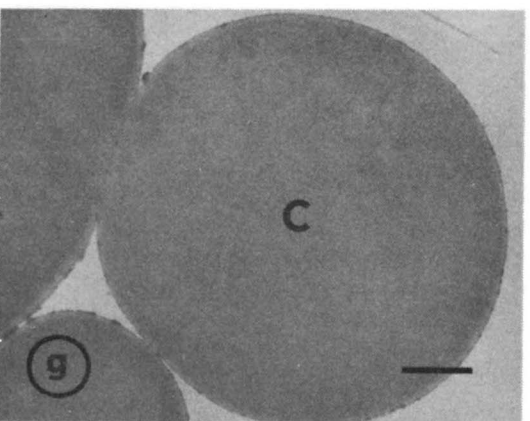
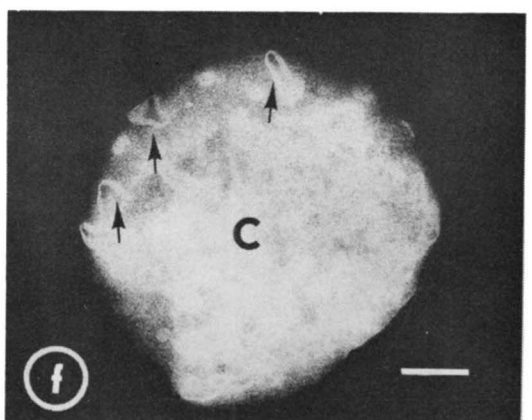
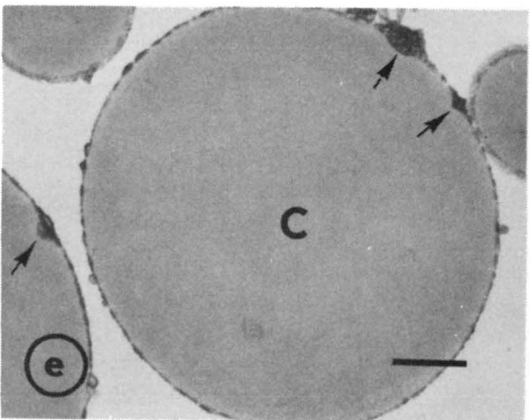
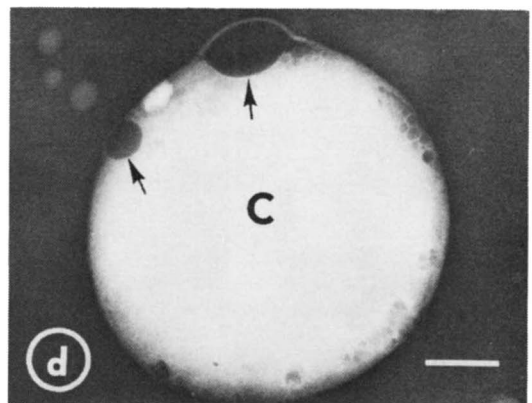
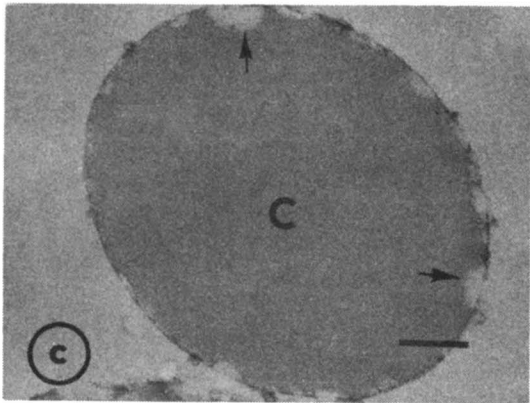
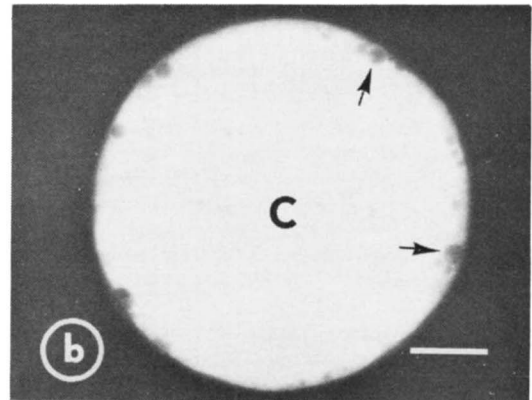
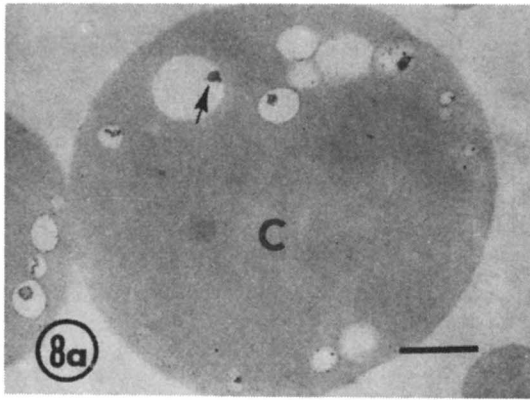


Fig. 8. Chylomicrons were incubated with lipoprotein lipase for 4 min in Tris-buffered medium, isolated by ultracentrifugation and suspended in different buffered albumin solutions (pH 8.1, 7.4, 5.5 and 3.0; see Table 1). Aliquots of each suspension were then fixed in OsO₄ at the indicated pH, centrifuged, and sectioned (Figs. 8-a, 8-c, 8-e and 8-g) or stained with sodium phosphotungstate at the indicated pH and examined as whole mounts (Figs. 8-b, 8-d, 8-f and 8-h). FFA accounted for 7% of the fatty acids present in these chylomicrons. BS/FA = 0.12; FFA/TFA = 0.19.

Fig. 8-a. OsO₄ at pH 8.1: electron-lucent circular areas are present within the chylomicron core (C). Some of the areas contain small patches of granular material (arrow). Compare with Fig. 8-b. × 100,000.

Fig. 8-b. Sodium phosphotungstate at pH 8.1: electron-opaque circular areas (arrows) are present within the electron-lucent chylomicron core (C). × 100,000.

Fig. 8-c. OsO₄ at pH 7.4: electron-lucent circular areas (arrows) are seen at the periphery of the chylomicron core (C). Compare with Fig. 8-d. × 90,000.

Fig. 8-d. Sodium phosphotungstate at pH 7.4: electron-opaque circular areas (arrows) are present peripherally within the electron-lucent chylomicron core (C). × 90,000.

Fig. 8-e. OsO₄ at pH 5.5: the chylomicron contains localized areas of osmiophilic deposits (arrows) at the periphery of the core (C). Compare with Fig. 8-f. See Fig. 10 for higher magnification. × 100,000.

Fig. 8-f. Sodium phosphotungstate at pH 5.5: curved electron-lucent areas (arrow) are present within the chylomicron core (C). See Fig. 11 for higher magnification of similar areas. × 90,000.

Fig. 8-g. OsO₄ at pH 3.0: the chylomicron core (C) is circular and homogeneously electron-opaque. Compare with Fig. 8-h. × 90,000.

Fig. 8-h. Sodium phosphotungstate at pH 3.0: the core lipid (C) is circular and electron-lucent. This chylomicron, which is partially depleted of core lipid, has redundant surface film (at arrow). × 80,000.

Lipolyzed chylomicrons: pH 6.0. Lipolyzed chylomicrons fixed with OsO₄ at pH 6.0 contained only a few electron-lucent areas, and those stained with sodium phosphotungstate at pH 6.0 contained few electron-opaque areas (Table 1).

Lipolyzed chylomicrons: pH 5.5. Lipolyzed chylomicrons fixed with OsO₄ at pH 5.5 were markedly different in structure from those fixed at pH 7.4 and 8.1 (compare **Figs. 6** and **8e** with **Figs. 4, 8a** and **8c**). Chylomicrons fixed at pH 5.5 did not have the electron-lucent areas but, instead, had numerous electron-opaque patches at the periphery of the core (**Figs. 6** and **8e**). Lamellar structures with a periodicity of 40 Å were present in the electron-opaque areas (**Fig. 10**). The lamellae were seen more easily in chylomicrons incubated with sodium veronal than in those incubated with Tris.

Lipolyzed chylomicrons stained with sodium phosphotungstate at pH 5.5 (**Fig. 7**) appeared mostly as electron-lucent spheres; only a few contained circular electron-opaque areas. Some of the chylomicrons had at the periphery curved electron-lucent areas (**Fig. 8f**) which consisted of lamellae with a periodicity of approximately 40 Å (**Fig. 11**).

Lipolyzed chylomicrons: pH 3.0. Lipolyzed chylomicrons fixed in OsO₄ at pH 3.0 were circular, homogeneous and free of both electron-lucent areas and electron-opaque patches (**Fig. 8g**). Chylomicrons stained with sodium phosphotungstate at pH 3.0 usually appeared as homogeneous electron-lucent spheres, but sometimes they were associated with redundant electron-opaque surface film (**Fig. 8h**).

Sodium oleate. A precipitate of sodium oleate was fixed with OsO₄ at pH 7.4, centrifuged and sectioned (**Fig. 12**). The sections contained numerous areas of lamellae consisting

of light and dark bands with a periodicity of 40 Å.

Triglyceride-depleted chylomicrons. Chylomicrons lipolyzed >95% (**Fig. 1**) were isolated as a thin floating layer by ultracentrifugation, and fixed with OsO₄ at pH 7.4. Sections of the floating layer showed numerous circular and crescentic structures ranging in diameter from 0.2 to 0.3 μm (**Fig. 13**). The structures contained concentric light and dark bands with a periodicity ranging from 45 to 60 Å.

DISCUSSION

These studies demonstrate structural changes induced in chylomicrons by the action of lipoprotein lipase in vitro. The chylomicrons studied were incubated with purified lipoprotein lipase in a medium containing limited albumin (BS/FA = 0.09–0.15) so that some of the FFA and partial glycerides formed during lipolysis would be retained in the chylomicrons (Table 3). Lipolyzed chylomicrons contained near the periphery of the triglyceride core small circular spaces, which at pH 8.1 and 7.4 stained with water soluble sodium phosphotungstate but not OsO₄. The spaces were not seen, however, in specimens prepared at pH 5.5 and 3.0. Rapid entry of sodium phosphotungstate into the spaces, at pH 8.1 and 7.4, indicates that the spaces communicated directly with the surrounding aqueous medium and thus contained water. As discussed below, the effect of lowering pH probably resulted from loss of charge on FFA lining the spaces and the consequent expulsion of water. Recent scanning electron micro-

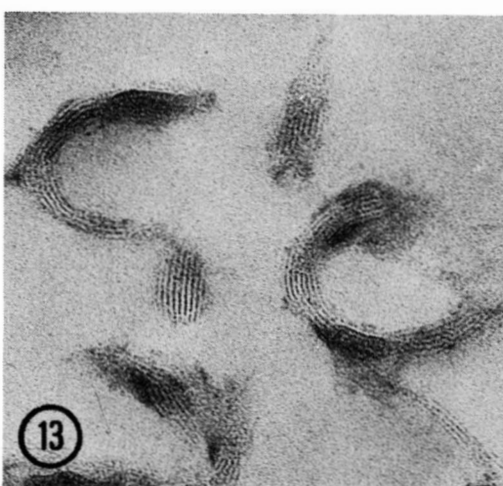
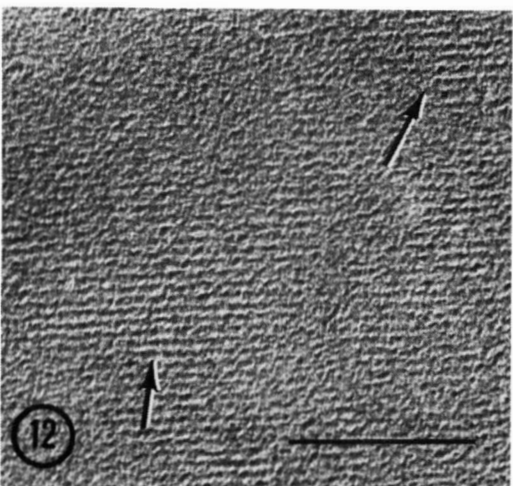
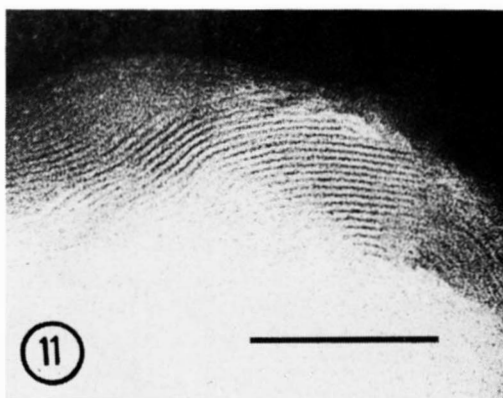
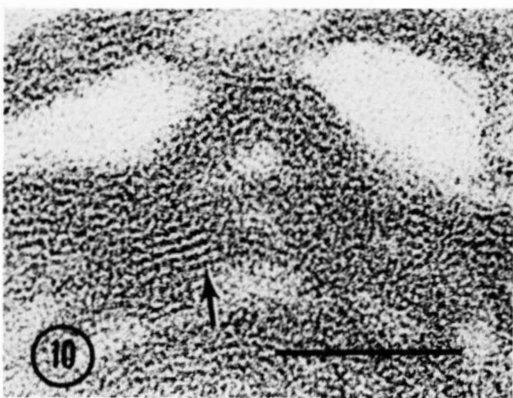
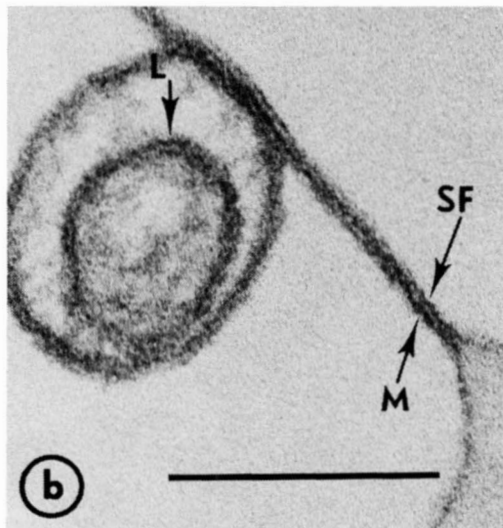
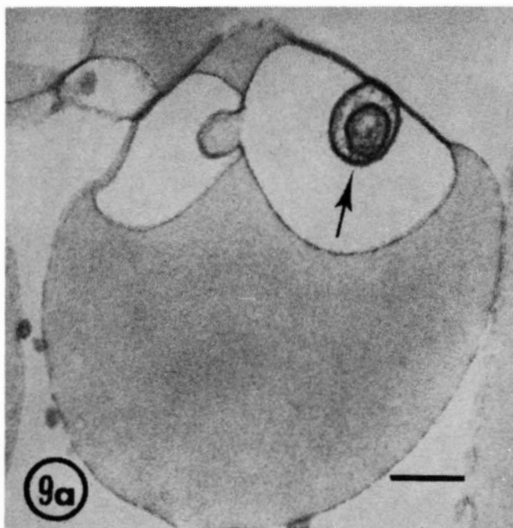


Fig. 9-a. Chylomicrons were incubated with lipoprotein lipase for 10 min in Tris-buffered medium, fixed with OsO₄ at pH 7.4, centrifuged and sectioned. Two large separate electron-lucent areas are present, one of which contains a whorled electron-opaque structure (arrow). BS/FA = 0.13; FFA/TFA = 0.18. × 90,000.

Fig. 9-b. Higher magnification of Fig. 9-a showing that the whorled structure consists of bilayered lamellae (L). Note that the outer layer of the lamella separating the electron-lucent area from the exterior is continuous with the chylomicron surface film (SF) and that the inner layer is continuous with the monolayer lining the electron-lucent area (M). Chylomicron core (C). × 360,000.

Fig. 10. Chylomicrons were incubated with lipoprotein lipase for 10 min in medium buffered with sodium veronal, separated from the incubation medium by ultracentrifugation, suspended in 0.1 M sodium cacodylate (albumin-free) solution at pH 5.5, fixed with OsO₄ at pH 5.5, centrifuged and sectioned. The lamellar structure seen has a periodicity of 40 Å (arrow). The dark bands represent the hydrophilic regions, and the light bands, the less osmiophilic hydrocarbon regions of lamellae of lipid bilayers. BS/FA = 0.15; FFA/TFA = 0.17. × 250,000.

Fig. 11. Chylomicrons were incubated with lipoprotein lipase for 4 min in Tris-buffered medium, separated from the incubation medium by ultracentrifugation, suspended in 4% albumin solution buffered with 0.1 M sodium cacodylate at pH 5.5, stained with sodium phosphotungstate at pH 5.5 and examined as whole mounts. The lamellar structure seen has a periodicity of 40 Å. The dark bands are due to the presence of water-soluble electron-opaque sodium phosphotungstate in the hydrophilic zones, and the light bands are due to absence of the stain in the hydrocarbon regions of lamellae of lipid bilayers. BS/FA = 0.12; FFA/TFA = 0.12. × 250,000.

Fig. 12. Sodium oleate at pH 7.4 was fixed with OsO₄ at pH 7.4, centrifuged and sectioned. The structure, which consists of lamellae of lipid bilayers, shows alternating light and dark bands with a periodicity of approximately 40 Å (arrow). × 250,000.

Fig. 13. Triglyceride-depleted chylomicrons. Chylomicrons incubated with lipoprotein lipase for 60 min in Tris-buffered medium were isolated by ultracentrifugation from a mixture of incubation medium and KBr (density = 1.25 g/ml), fixed with OsO₄ at pH 7.4, centrifuged and sectioned. These curved figures show light and dark banding with a periodicity of 60 Å. FFA accounted for 90% of the fatty acids present. BS/FA = 1.00; FFA/TFA = 0.97. × 100,000.

scopic studies¹ of lipolyzed chylomicrons fixed with OsO₄ showed that specimens prepared at pH 7.4 had multiply indented surfaces whereas those prepared at pH 5.5 had smooth surfaces. Sections of lipolyzed chylomicrons that had been prepared for scanning electron microscopy showed that the surface indentations correspond to the electron-lucent areas seen in chylomicrons prepared for transmission electron microscopy (Figs. 4, 8a, 8c and 9a). It was concluded that vacuum dehydration during preparation of specimens for scanning electron microscopy caused indentations of the chylomicron surface by rupturing the lamellae which separated the aqueous spaces from the exterior (Fig. 9a).

The aqueous spaces in lipolyzed chylomicrons contained aggregates of osmiophilic material and whorls of bilayered lamellae (Figs. 8a and 9a). The spaces were replaced with lamellar structures, with a periodicity of about 40 Å, in specimens prepared at pH 5.5 (Figs. 8e, 8f, 10 and 11), and the lamellae, as well as the spaces, were absent in those prepared at pH 3.0 (Figs. 8g and 8h). Lamellar structures with a periodicity of 40 Å can be found in a large variety of lipids containing long chain fatty acids (11). The increased incidence of bilayered lamellae in lipolyzed chylomicrons prepared at pH 5.5 (Figs. 8e, 8f, 10 and 11), and their absence at pH 3.0 (Fig. 8g) indicate that one or more of the lipids in the chylomicrons underwent marked change when pH was reduced. Of the various acyl lipids in lipolyzed chylomicrons, FFA, because of the free carboxyl group, probably undergo the greatest change with pH (11, 12).

¹ E. J. Blanchette-Mackie and R. O. Scow, Scanning electron microscopic study of chylomicrons incubated with lipoprotein lipase. *Anat. Rec.* In press.

If FFA in chylomicrons had the same dissociation constant as fatty acids in bulk phase, $pK_a = 4-5$ (13, 14), they would be present in chylomicrons primarily as soaps at pH 8.1 and 7.4, as a mixture of soaps and acids at pH 5.5, and as acids at pH 3.0. However, FFA are amphiphilic (12) and probably are located in chylomicrons in the lipid interface between core triglyceride and incubation medium (pH 8.1). Thus, they would have a pK_a 3-4 units higher than that of fatty acids in bulk phase (13, 14) and would be present in chylomicrons as a mixture of soaps and acids at pH 8.1 and 7.4, and mostly as acids at pH 5.5 and 3.0. Soaps of long chain fatty acids are slightly soluble in water and insoluble in triglyceride, whereas the acids are soluble in triglyceride and insoluble in water (12, 15). Mixtures of fatty acids and soaps form acid-soap complexes which are insoluble in both solvent systems (15-17). Soaps and acid-soap complexes, but not fatty acids, form bilayered lamellae under certain conditions (12, 17, 18).

The absence of aqueous spaces and the increased incidence of lamellae in lipolyzed chylomicrons processed at pH 5.5, and the absence of both aqueous spaces and lamellae at pH 3.0 suggest that FFA are associated with the aqueous spaces. The FFA, as discussed above, probably were located in the lipid interface between triglyceride and water, in the monolayer lining the aqueous spaces as well as in the chylomicron surface film. The lamellar structures seen in lipolyzed chylomicrons prepared at pH 5.5 (Figs. 8c, 8f, 10 and 11) probably formed when monolayers and whorls of lamellae in the aqueous spaces (Figs. 9a and 9b) were condensed during the movement of water from the spaces, and the latter probably resulted from decreased charge on FFA lining the aqueous spaces (11, 12, 19). Lamellae were not visible in specimens prepared at pH 3.0 (Fig. 8g) perhaps due to dissolution of

protonated fatty acids into core triglyceride. The lamellar structures in triglyceride-depleted chylomicrons (Fig. 13) also probably formed as the result of water loss, while the specimens were being centrifuged in hypertonic salt solution (2.4 M KBr).

Electron microscopic studies have shown that soaps fixed with OsO_4 have a lamellar structure (20–24) similar to that found in lipolyzed chylomicrons fixed with OsO_4 (Fig. 10). The lamellae, according to x-ray diffraction studies, consist of bilayers of fatty acids arranged with their hydrocarbon chains extending inwardly (18, 21). Results of several studies indicate that the light and dark bands seen in electron micrographs of OsO_4 -fixed soaps (Fig. 12) can be related to the hydrocarbon and polar regions of lamellae (21, 24). Although OsO_4 reacts with double bonds of fatty acids and is essential for fixation of structure, the small amounts present in the hydrocarbon regions are not easily detected with the electron microscope (20, 21, 24). The hydrophilic polar regions, in contrast, concentrate OsO_4 and appear as dark bands in electron micrographs (20, 21, 24). The lamellae seen in lipolyzed chylomicrons stained with sodium phosphotungstate at pH 5.5 (Fig. 11) closely resemble those seen in chylomicrons fixed with OsO_4 at pH 5.5 (Fig. 10) and in sodium oleate fixed with OsO_4 at pH 7.4 (Fig. 12). The periodicity of the lamellae is 40 Å, and the hydrophilic polar regions, due to the presence of electron-opaque sodium phosphotungstate in those regions, appear as dark bands, while the hydrocarbon regions appear as light bands (Fig. 11). The light bands in specimens stained with sodium phosphotungstate are more distinct and wider than those in specimens fixed with OsO_4 (compare Fig. 11 with Figs. 10 and 12), perhaps because small amounts of osmium are present in the hydrocarbon regions of specimens fixed with OsO_4 (12). The lamellar structures seen in lipolyzed and triglyceride-depleted chylomicrons probably are composed, at least in part, of soaps and acid-soap complexes of fatty acids.

Monoglyceride and diglyceride accumulated in chylomicrons incubated with lipoprotein lipase in a medium containing limited albumin (Table 3). Since both are more amphiphilic than triglyceride, they too probably localized in the triglyceride–water interface. Due to the positional specificity of lipoprotein lipase (25), diglyceride is an intermediate product and 2-monoglyceride, if not isomerized, is a final product of the action of the enzyme on triglyceride. Thus, it is likely that diglyceride was present in chylomicrons mostly in the surface film, associated with the enzyme, and that monoglyceride was present in both the surface film and the monolayer lining the aqueous spaces.

The finding that the aqueous spaces in lipolyzed chylomicrons communicate directly with the surrounding aqueous medium and the possibility that the spaces are lined by a monolayer of FFA and monoglyceride suggest that the monolayer may be an extension of the surface film, although different in composition, formed by the accumulation of lipolytic products in the triglyceride–water interface. Thus, products of lipolysis probably moved by lateral diffusion (26) from the site of enzyme action to the monolayer lining the aqueous space, and lipolysis continued as long as the monolayer could expand. The finding of bilayered lamellae in the aqueous

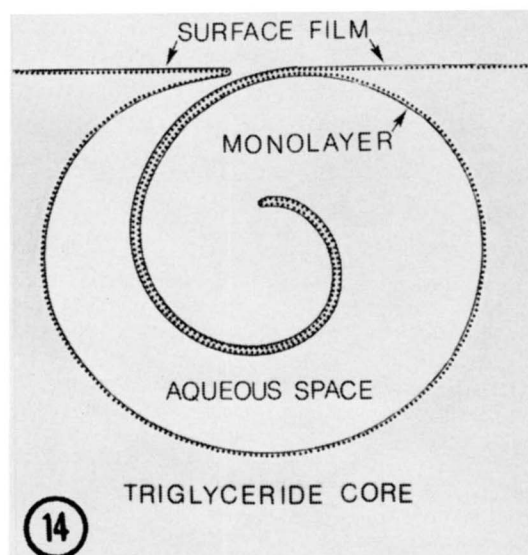


Fig. 14. Schematic representation of the formation of bilayered lamellae in the aqueous spaces of lipolyzed chylomicrons.

spaces of chylomicrons fixed with OsO_4 at pH 7.4 (Fig. 9b) suggests that the monolayer extended itself by forming a spiralling fold within the aqueous space (Fig. 14). It is evident, from the above, that hydrolysis of triglyceride to FFA and partial glycerides results in the formation of acyl products which can easily locate and move within the interfacial plane between triglyceride and water.

The above studies were made primarily in chylomicrons that had been incubated with purified lipoprotein lipase in a medium containing limited albumin (BS/FA = 0.09–0.15). Consequently, products of lipolysis (FFA and partial glycerides) and water accumulated and produced marked structural changes in the chylomicrons (Figs. 9a and b). FFA accumulated also when excess albumin was available (BS/FA = 1.2–1.4), and formed bilayered lamellae in chylomicrons reduced 95% in triglyceride content (Fig. 13). Whether lipolytic products accumulate in chylomicrons lipolyzed in capillaries, in vivo (27, 28) or in perfused tissues (29–31), has not been studied. **66**

The authors are grateful to Mr. David B. Winter and Mrs. T. Ruth Fleck for expert technical assistance, and to Drs. Louis C. Smith and Donald M. Small for valuable discussions of the study.

Manuscript received 3 March 1975 and in revised form 16 June 1975; accepted 8 September 1975.

REFERENCES

1. Blanchette-Mackie, E. J., and R. O. Scow. 1973. Effects of lipoprotein lipase on the structure of chylomicrons. *J. Cell Biol.* **58**: 689–708.
2. Egelrud, T., and T. Olivecrona. 1972. The purification of a lipoprotein lipase from bovine skim milk. *J. Biol. Chem.* **247**: 6212–6217.

3. Egelrud, T., and T. Olivecrona. 1973. Purified bovine milk (lipoprotein) lipase: activity against lipid substrates in the absence of exogenous serum factors. *Biochim. Biophys. Acta.* **306**: 115-127.
4. Havel, R. J., C. J. Fielding, T. Olivecrona, V. G. Shore, P. E. Fielding, and T. Egelrud. 1973. Cofactor activity of protein components of human very low density lipoprotein in the hydrolysis of triglycerides by lipoprotein lipase from different sources. *Biochemistry.* **12**: 1828-1833.
5. Scow, R. O., Y. Stein, and O. Stein. 1967. Incorporation of dietary lecithin and lysolecithin into lymph chylomicrons in the rat. *J. Biol. Chem.* **242**: 4919-4924.
6. Chernick, S. S., and M. Novak. 1970. Effect of insulin on FFA mobilization and ketosis in fasting pregnant rats. *Diabetes.* **19**: 563-570.
7. Stein, O., R. O. Scow, and Y. Stein. 1970. FFA-³H uptake by perfused adipose tissue: electron microscopic autoradiographic study. *Amer. J. Physiol.* **219**: 510-518.
8. Hamosh, M., T. R. Clary, S. S. Chernick, and R. O. Scow. 1970. Lipoprotein lipase activity of adipose and mammary tissue and plasma triglyceride in pregnant and lactating rats. *Biochim. Biophys. Acta.* **210**: 473-482.
9. Rapport, M. M., and M. Alonzo. 1959. Photometric determination of long chain fatty acids in plasma and tissues. *J. Biol. Chem.* **217**: 195-198.
10. Blanchette-Mackie, E. J., and R. O. Scow. 1971. Sites of lipoprotein lipase activity in adipose tissue perfused with chylomicrons. Electron microscope cytochemical study. *J. Cell Biol.* **51**: 1-25.
11. Dervichian, D. C. 1964. The physical chemistry of phospholipids. *Prog. Biophys. Mol. Biol.* **14**: 263-342.
12. Small, D. M. 1968. A classification of biologic lipids based upon their interaction in aqueous systems. *J. Amer. Oil Chem. Soc.* **45**: 108-117.
13. Goddard, E. D. 1974. Ionizing monolayers and pH effects. *Adv. Colloid Interface Sci.* **4**: 45-78.
14. Mattson, F. H., and R. A. Volpenhein. 1966. Enzymatic hydrolysis at an oil/water interface. *J. Amer. Oil Chem. Soc.* **41**: 286-289.
15. Ekwall, P., and L. Mandell. 1968. Solutions of alkali soaps and water in fatty acids I. Region of existence of the solutions. *Kolloid Z. and Z. Polym.* **233**: 938-944.
16. Friberg, S., L. Gezelius, and I. Wilton. 1971. Influence of acid soap interactions on the solubility of soaps in triglycerides. *Chem. Phys. Lipids.* **6**: 364-372.
17. Kung, H. C., and E. D. Goddard. 1969. Molecular association in fatty acid potassium soap systems. II. *J. Colloid Interface Sci.* **29**: 242-249.
18. Luzzati, V. 1968. X-ray diffraction studies of lipid-water systems. *In Biological Membranes, Physical Fact and Function.* D. Chapman, editor. Academic Press, London, 71-123.
19. Adams, N. K. 1944. *The Physics and Chemistry of Surfaces.* Oxford University Press, London. 22.
20. Corkill, J. M., and J. F. Goodman. 1969. Interaction of non-ionic surface-active agents with water. *Adv. Colloid Interface Sci.* **2**: 297-330.
21. Stoeckenius, W. 1959. An electron microscope study of myelin figures. *J. Biophys. and Biochem. Cytol.* **5**: 491-500.
22. Stoeckenius, W. 1960. Osmium tetroxide fixation of lipids. *Proc. European Regional Conf. Electron Microscopy, Delft,* **2**: 716-720.
23. Stoeckenius, W., J. H. Schulman, and L. M. Prince. 1960. The structure of myelin figures and microemulsions as observed with the electron microscope. *Kolloid Z.* **169**: 170-180.
24. Trurnit, H. J., and G. Schidlovsky. 1960. Thin cross-sections of artificial stacks of monomolecular films. *Proc. European Regional Conf. Electron Microscopy, Delft,* **2**: 721-725.
25. Nilsson-Ehle, P., T. Egelrud, P. Belfrage, T. Olivecrona, and B. Borgström. 1973. Positional specificity of purified milk lipoprotein lipase. *J. Biol. Chem.* **248**: 6734-6737.
26. McConnell, H. M., P. Devaux, and C. Scandella. 1972. Lateral diffusion and phase separations in biological membranes. *In Membrane Research.* C. F. Fox, editor. Academic Press, New York. 27-37.
27. Redgrave, T. G. 1970. Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. *J. Clin. Invest.* **49**: 465-471.
28. Bergman, E. N., R. J. Havel, B. M. Wolfe, and T. Bohmer. 1971. Quantitative studies of the metabolism of chylomicron triglycerides and cholesterol by liver and extrahepatic tissues of sheep and dogs. *J. Clin. Invest.* **50**: 1831-1839.
29. Scow, R. O., C. R. Mendelson, O. Zinder, M. Hamosh, and E. J. Blanchette-Mackie. 1973. Role of lipoprotein lipase in the delivery of dietary fatty acids to lactating mammary tissue. *In Dietary lipids and postnatal development.* C. Galli, G. Jacini, and A. Pecile, editors. Raven Press, New York. 91-114.
30. Fielding, C. J., and J. M. Higgins. 1974. Lipoprotein lipase: comparative properties of the membrane-suspended and solubilized enzyme species. *Biochemistry.* **13**: 4324-4330.
31. Noel, S. P., P. J. Dolphin, and D. Rubinstein. 1975. An in vitro model for the catabolism of rat chylomicrons. *Biochem. Biophys. Res. Comm.* **63**: 764-772.