

SITES OF LIPOPROTEIN LIPASE ACTIVITY IN ADIPOSE TISSUE PERFUSED WITH CHYLOMICRONS

Electron Microscope Cytochemical Study

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ABSTRACT

Lipoprotein lipase activity was studied in rat parametrial adipose tissue perfused with chylomicrons and in gelatin blocks containing postheparin plasma and chylomicrons. The tissues and blocks were fixed in glutaraldehyde and incubated in 0.035 M CaCl₂-0.1 M Tris medium (pH 8.3) at 38°C. The doubly labeled chylomicron triglycerides (glycerol-³H and palmitate-¹⁴C) in the tissues and blocks were hydrolyzed during incubation to free fatty acids (FFA) and the FFA remained in the specimens; hydrolysis was inhibited by 0.004 M diethyl paranitrophenyl phosphate (E-600). Incubated blocks and tissue were treated with 0.05 M Pb(NO₃)₂, postfixed in OsO₄, dehydrated with acetone, embedded in Epon, and examined by electron microscopy. The incubated blocks contained electron-lucent areas and granular and laminar precipitates at sites of hydrolysis. Similar precipitates were found in incubated tissue, within vacuoles and microvesicles of capillary endothelium, and in the subendothelial space (between the endothelium and pericytes), but not in the capillary lumen or in or near fat cells. The cytochemical reaction was greatly reduced in blocks and tissues incubated with E-600. It is concluded that plasma glycerides are hydrolyzed by lipoprotein lipase in capillary endothelial cells and in the subendothelial space of adipose tissue and that glycerides cross the endothelial cells within a membrane-bounded system.

INTRODUCTION

Fatty acids are transported in the blood stream as triglyceride, in the form of chylomicrons and very low density (or pre- β) lipoproteins, and as free fatty acids (FFA) (11, 12, 41, 42). There is considerable evidence now that uptake of blood triglyceride-fatty acids by most tissues (adipose, mammary, cardiac, skeletal muscle) involves hydrolysis of the triglyceride to FFA and that hydrolysis is catalyzed and regulated by lipoprotein lipase (12, 31, 41). Triglyceride uptake and lipoprotein lipase activity in adipose tissue are both decreased by fasting and diabetes, and increased by refeeding and insulin administration (2, 4, 8, 17, 18, 31, 35, 37, 38). Results obtained in early perfusion studies of adipose tissue suggested that two-thirds of the triglyceride removed were hydrolyzed completely to FFA and glycerol, and that the rest was retained intact in the tissue (36). Subsequent studies, however, showed that more than 90% of the triglyceride taken up by

this tissue is immediately hydrolyzed (41). Although the site of action of lipoprotein lipase has not been demonstrated, studies in perfused adipose tissue have shown that most of the triglyceride is hydrolyzed after it has been removed from the blood stream (35, 36).

Studies of lipoprotein lipase activity in fat cells and in stromal-vascular cells isolated from adipose tissue showed that the enzyme activity was present mostly in fat cells (6, 27, 34). However, the amount of activity recovered accounted for only 14% of that present initially in the tissue (6). The rapid release of lipoprotein lipase to the blood stream by adipose tissue perfused with heparin suggests that the enzyme is also present in or near the vascular bed (16, 35). Histochemical studies of adipose tissue of fed mice have demonstrated lipolytic activity in the capillary bed (26). Electron microscope studies of adipose tissue from animals fed lipid-rich diets showed chylomicrons present in the capillary lumen and attached to the luminal surface of the endothelium, but neither in endothelial cells nor beyond the capillary wall (46, 47). On the basis of these studies, it was suggested that partial hydrolysis of chylomicron triglyceride might occur at the point of attachment of chylomicrons to the endothelial cell and that hydrolysis of the resultant product might occur within the capillary endothelium and in the extravascular space (36).

Electron microscope studies of mammary tissue of lactating rodents showed chylomicrons and lipid particles of artificial triglyceride emulsions attached to the luminal surface of the endothelium and in endothelial vacuoles and multivesicular bodies, but not in pinocytotic vesicles, in intercellular gaps, or on the outer surface of the endothelium (39). Cytochemical studies of mammary tissue demonstrated hydrolysis of chylomicrons and triglyceride particles in the capillary lumen, presumably by lipoprotein lipase, but not in the endothelium nor outside the capillaries (39).

The present experiments were designed to determine sites of hydrolysis of chylomicron-triglyceride by lipoprotein lipase in adipose tissue and how glyceride-fatty acids cross the capillary wall. The tissue was perfused with doubly labeled chylomicrons so that cytochemical and ultrastructural findings could be related to biochemical events. The cytochemical technique used for demonstrating lipoprotein lipase activity (39) was

validated with morphological and biochemical studies in plasma-gelatin blocks containing the enzyme and doubly labeled chylomicrons.

METHODS

Animals

Adult female Charles River rats weighing 170–190 g each and fed *ad libitum* Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) were used in these experiments.

Preparation of Chylomicrons

Doubly labeled chylomicrons were isolated from thoracic duct lymph collected from fasted rats which had been tube-fed corn oil containing palmitic-1-¹⁴C acid (55 mCi/mole, CFA 23, Batches 44 and 48, Nuclear-Chicago, Des Plaines, Ill.) and trioleoylglycerol-2-³H (143–168 mCi/mole, TRA 172, Batches 4 and 5, Amersham-Searle Corp., Des Plaines, Ill.) (43). The chylomicrons were suspended in 4% albumin solution (bovine plasma albumin powder [Fraction V, Armour Pharmaceutical Co., Chicago, Ill., Lot E-29907] in Tyrode's solution) at a triglyceride concentration of 70–110 mM. They were stored at 4°C and used within 5 days after being collected. The chylomicron suspension was diluted to a triglyceride concentration of 20–25 mM on the day of the experiment with serum prepared from perfusing fluid (see below).

Chylomicrons suspended in albumin solution were prepared for electron microscope study as follows. They were fixed for 30 min in cold 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and centrifuged in a Beckman Microfuge at 12,000 rpm for 1 min (Beckman Instruments, Inc., Fullerton, Calif.). The pellet containing the chylomicrons was then postfixed with OsO₄, dehydrated, embedded in Epon, and sectioned by the procedures used for tissue (see below).

Perfusion Technique

The left parametrial fat body of the rat was perfused by the technique of Robert and Scow (28). The fat-pad donor was anesthetized with ether while the fat body and its blood supply, the uterine artery and vein, were isolated and prepared for transfer to the tissue chamber; this took about 15 min. Transfer of the tissue, cannulation of the blood vessels, and reestablishment of blood flow through the tissue were completed in less than 1 min. The fat pad was floated in the tissue chamber in 2–3 ml of serum prepared from the perfusing fluid.

The perfusing apparatus, described in detail elsewhere (28, 40, 44), consisted of reservoir-oxygenator,

pulsating-type pump assembly (150 pulses/min), filter, manometer, injection site, and tissue chamber. The temperature of the tissue chamber and blood entering the tissue was maintained at 38°–39°C. The blood was oxygenated in a rotating flask with a gas mixture of 95% O₂–5% CO₂. Arterial pressure was maintained at 85–90 mm Hg and venous pressure at less than 1 mm H₂O. Venous blood was collected in ice-cold 5-ml graduated centrifuge tubes.

The perfusing fluid consisted of defibrinated rat blood, from fed animals, diluted 1:10 with a 4% solution of bovine plasma albumin (Fraction V, Armour, Lot E-29907) in modified Tyrode's solution (28). It contained glucose (0.5 mg/ml), penicillin B (50 U/ml), and dihydrostreptomycin sulfate (50 µg/ml).

The tissues were perfused for 10 min with chylomicron-free blood before being perfused with blood containing chylomicrons. A suspension of chylomicrons containing 20–25 mM triglyceride was then injected into the arterial blood stream at a constant rate (28). The rate of injection was adjusted to produce a triglyceride concentration of 3–5 mM in the arterial blood. Earlier studies showed that maximal hydrolysis of chylomicron triglyceride by perfused adipose tissue occurred at a concentration of 5 mM (36). Perfusion of the fat pad was stopped while chylomicrons were flowing through the tissues. The fat pad was immediately transferred to 0.1 M sodium cacodylate buffer solution (pH 7.4) at < 4°C and divided into two portions; the distal portion, weighing 100–150 mg, was taken for biochemical and cytochemical studies, and the proximal portion, which included the large uterine vessels and tributaries to the uterus, was discarded (41, 44). The distal portion was then quickly cut into several pieces, of which one was put into ice-cold chloroform:methanol (2:1) for chemical analyses and the rest were put into ice-cold (< 4°C) glutaraldehyde-cacodylate buffer (see below) for incubation studies.

Lipoprotein Lipase Activity in Fixed Tissue

The pieces of tissue used for studies of lipoprotein lipase activity were fixed 30 min at < 4°C in 3% glutaraldehyde solution buffered with 0.1 M sodium cacodylate (pH 7.4) and containing 0.0045 M CaCl₂, and washed 1 hr at < 4°C in 0.1 M sodium cacodylate buffer (pH 7.4). They were then cut into blocks, 1 × 1 × 1 mm, and incubated at 38°C in 0.035 M CaCl₂–0.1 M Tris (tris[hydroxymethyl]amino methane) medium (pH 8.3). Blocks of tissue were taken at the beginning and at the end of incubation for morphological and biochemical analyses. In a few experiments blocks were sectioned at 30–50 µ on a cryostat (International Equipment Company, Needham Heights, Mass., Model CTI) before being incubated.

The tissue specimens taken for electron microscopy were rinsed in cold 0.1 M sodium cacodylate buffer, treated 15 min with 0.06 M Pb(NO₃)₂–0.1 M sodium cacodylate solution (pH 6.8) at 24°C, and postfixed 2 hr in ice-cold 2% OsO₄ in 0.1 M sodium cacodylate buffer containing 0.0045 M CaCl₂ (pH 7.4). The specimens were then dehydrated rapidly in graded acetone (one change of 75%, two of 95%, and one of 100% at 4°C within 5 min, and two changes of 100% at 24°C within 20 min) and infiltrated at 24°C with complete Epon mixture (22) (15 hr in 67% Epon in acetone, and 8 hr in 100% Epon). The Epon blocks were cured at 45°C for 1 day and at 65°C for 2 days. Thin sections were cut on a Reichert ultramicrotome with a diamond knife and examined in a Philips EM 300 electron microscope. Some of the sections were stained with uranyl acetate and lead citrate (45).

Lipoprotein Lipase Activity in Plasma-Gelatin Blocks

Plasma-gelatin blocks containing doubly labeled chylomicrons and lipoprotein lipase activity were prepared as follows. 2.5 ml of plasma, either control or postheparin, 2.5 ml of 4% albumin solution in 0.1 M Tris buffer (pH 8.3), and 0.25 ml of a suspension of doubly labeled chylomicrons (50 mM triglyceride) were gently mixed together with a Pasteur pipette at 24°C for 1 min and quickly chilled to 0°–4°C. 1 ml of this mixture was then mixed with 1 ml of warm (40°C) 8% gelatin solution for less than 30 sec and immediately pipetted into cold (0°C) flat embedding molds (3 × 4 × 12 mm) (Silicone-rubber, Ladd Research Industries, Inc., Burlington, Vt.). As soon as the mixture had solidified, usually within 1 min, the blocks were removed from the molds, fixed 30 min in cold (2°–4°C) 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (7.4), and washed 60–80 min in cold (2°–4°C) 0.1 M sodium cacodylate buffer (pH 7.4). The blocks were used within 2 hr for incubation studies described in Tables I–III. Most incubations were made in duplicate so that blocks and media in one set could be used for biochemical analyses and blocks in the other set could be used for morphological studies. The procedures used for preparing plasma-gelatin blocks for electron microscope visualization were the same as those used for tissue (see above). Most of the blocks were treated 15 min with 0.06 M Pb(NO₃)₂–0.1 M sodium cacodylate solution before osmication.

Control plasma with low lipoprotein lipase activity was prepared from blood drawn from the aorta of a fed rat anesthetized with ether; the blood was drawn into a syringe containing 1.0 mg of heparin. Postheparin plasma with high lipoprotein lipase activity (30) was prepared from blood drawn from an anesthetized rat given 0.5 mg of heparin intra-

TABLE I
Hydrolysis of Chylomicron-Glyceride by Lipoprotein Lipase during Preparation and Incubation of Plasma-Gelatin Blocks: Effect of E-600

Specimen	Time elapsed	Incubation		No. of expts.	Distribution of glycerol- ³ H†				FFA released from glyceride**
		Addition	Time		TG	DG	MG	Free glycerol	
	min		hr		% total	% total	% total	% total	% glyceride-fatty acids
Chyl*	—	—	—	—	94	4	2	0	0
Chyl + PHP‡	0	—	—	1	83	5	11	1	7.3
Blocks									
Unfixed§	8-20	—	—	3	74	4	15	7	15.7
Fixed	120	None	0	2	71	3	12	14	20.4
“	180	“	1	2	33	2	5	60	60.3
“	240	“	2	2	15	2	6	77	79.0
“	180	E-600	1	2	69	2	4	23	25.6
“	240	“	2	2	68	3	7	22	25.0

* Chyl = doubly labeled chylomicrons (glyceride-glycerol-³H and palmitic acid-¹⁴C).

‡ Chyl and PHP (postheparin plasma + 4% albumin solution) were mixed for 1 min at 24°C, chilled immediately to 2°C, and a sample was taken 1 min later.

§ Chyl + PHP were mixed for <30 sec with warm (38°C) 8% gelatin solution, poured into ice-cold molds, and chilled immediately to 0°-2°C; blocks were taken for analyses 1 min later.

|| Blocks were fixed 30 min in 3% glutaraldehyde at 2°-4°C, washed 60-80 min in 0.1 M sodium cacodylate at 2°-4°C, and then incubated in 0.035 M CaCl₂-0.1 M Tris medium (pH 8.3) at 38°C. E-600 = 0.004 M diethyl paranitrophenyl phosphate was added to incubation medium.

† TG = triglyceride, DG = diglyceride, and MG = monoglyceride.

** Calculated from changes in glyceride content. About 1.2% of the fatty acids in chylomicrons were FFA.

venously 5 min before the blood was taken; this blood was also drawn into a syringe containing 1.0 mg of heparin.

Hydrolysis of chylomicron triglyceride to monoglyceride and FFA occurred when chylomicrons were being mixed with postheparin plasma at 24°C (Table I), and stopped when the mixture was chilled to 0°-2°C. Hydrolysis resumed, with further breakdown of triglyceride and release of glycerol and FFA, when the chylomicron-postheparin plasma mixture was mixed with warm gelatin solution (Table I), and stopped when the mixture was chilled and solidified at 0°-2°C. Further hydrolysis of glyceride was negligible as long as the blocks were maintained at a temperature less than 4°C (Tables I and II). Monoglycerides accounted for 12% of the glycerides in fixed blocks at the start of the incubation studies, whereas they accounted for only 2% of those in chylomicrons (Table I). This suggests that chilling to 0°-2°C has an inhibitory effect on hydrolysis of triglyceride and monoglyceride but not on that of diglyceride.

Biochemical Analyses

Lipids in chylomicrons, adipose tissue, and plasma-gelatin blocks were extracted into chloroform by the

method of Folch et al. (10), and lipids in blood and incubation media were extracted into hexane by a modification (5) of the method of Dole and Meinertz (7). The lipid extracts in some experiments were separated into glyceride and FFA fractions by the method of Borgstrom (3), or into neutral lipid (triglyceride, diglyceride, and monoglyceride) and FFA fractions by thin layer chromatography (44). The solutions used to postfix, dehydrate, infiltrate, and embed plasma-gelatin blocks were also analyzed for lipid-¹⁴C (Table III). The OsO₄-sodium cacodylate solutions were treated with ascorbic acid before the lipids were extracted into hexane (5). The acetone washes were mixed with a small amount of water, heated to 70°C to evaporate the acetone, and treated with ascorbic acid before the lipids were extracted into hexane (5). Epon-acetone and Epon were heated on a steam bath to evaporate the acetone and 1 ml of the residue was added directly to scintillating solution.

The lipid fractions were dissolved in 15 ml of toluene containing 4.2% Liquifluor (New England Nuclear, Pilot Chemicals Division, Boston, Mass., Cat. No. NEF-903) for measurement of ³H and ¹⁴C content in a liquid scintillation spectrometer (Packard Tricarb Model 314-EX or 3320). The gain and

TABLE II

Hydrolysis of Chylomicron-Glyceride in Glutaraldehyde-Fixed Plasma-Gelatin Blocks Incubated in Different Media

Gelatin blocks (3 × 4 × 10 mm) containing chylomicrons, albumin, and either control or postheparin plasma were prepared as described in Methods. The chylomicrons contained triglyceride labeled with glycerol-³H and palmitic acid-¹⁴C. The blocks were fixed for 30 min in cold 3% glutaraldehyde, washed 60 min in cold 0.1 M cacodylate buffer (pH 7.4), and then incubated as shown for 1-3 hr. In experiments with postheparin I plasma each block was incubated in 3 ml of media. In the other experiments, with postheparin II and control II plasmas, each incubation vial contained two blocks in 2 ml of media. Media concentrations were 0.035 M CaCl₂-0.1 M Tris (tris[hydroxymethyl]-amino methane), 0.1 M Tris, and 0.03 M Pb(NO₃)₂-0.1 M sodium cacodylate.

Plasma	Incubation medium	pH	Temperature	No. of expts.	Chylomicron-glyceride hydrolyzed		
					1 hr	2 hr	3 hr
			°C		% of glyceride*		
Postheparin I	CaCl ₂ -Tris	8.3	2-4	2	—	3	—
	CaCl ₂ -Tris	8.3	38	2	27	45	—
	Tris	8.3	38	2	—	24	—
Postheparin II	CaCl ₂ -Tris	8.3	38	1	57	—	99
	Tris	8.3	38	1	30	—	74
	Pb(NO ₃) ₂ -sodium cacodylate	6.8	38	1	15	—	53
Control II	CaCl ₂ -Tris	8.3	38	1	6	—	9
	Tris	8.3	38	1	1	—	5
	Pb(NO ₃) ₂ -sodium cacodylate	6.8	38	1	3	—	6

* Glyceride present at start of incubation.

discriminators were set so that ³H was counted with a relative efficiency of 100% in channel A and totally discriminated from channel B, and ¹⁴C was counted with a relative efficiency of 100% in channel B and 20% in channel A. The counts measured in channel A were corrected for those due to ¹⁴C. Appropriate internal standards were used to determine corrections for quench caused by discolored lipid extracts, acetone, and Epon.

Earlier studies of parametrial fat pads perfused with doubly labeled chylomicrons showed that more than 70% of the FFA released and less than 10% of the glycerol released from chylomicron triglyceride were incorporated into tissue triglyceride, and that the ratio of labeled glycerol to labeled fatty acids in newly synthesized triglyceride was usually less than 10% of that in chylomicrons (36, 41). For the purpose of calculation in the present study, it was assumed that incorporation of chylomicron triglyceride-glycerol into tissue triglyceride was negligible and that the ratio of labeled glycerol to labeled fatty acids in tissue triglyceride was zero. The chylomicrons used in the present study contained triglyceride labeled with glycerol-³H and palmitic acid-¹⁴C. Conse-

quently, the relative amounts of fatty acid-¹⁴C in chylomicron triglyceride remaining in the tissue, and in newly synthesized glyceride were calculated from the amount of ¹⁴C in the tissue glyceride fraction, and the ratios of ³H to ¹⁴C in the tissue glyceride fraction and in chylomicrons:

Chylomicron-¹⁴C in tissue = glyceride-¹⁴C

$$\times \frac{{}^3\text{H}/{}^{14}\text{C in glyceride}}{{}^3\text{H}/{}^{14}\text{C in chylomicrons}}$$

New triglyceride-¹⁴C = glyceride-¹⁴C

$$\times \left(1 - \frac{{}^3\text{H}/{}^{14}\text{C in glyceride}}{{}^3\text{H}/{}^{14}\text{C in chylomicrons}} \right)$$

The relative amount of chylomicron-glyceride hydrolyzed in plasma-gelatin blocks was calculated from the ratio of ³H to ¹⁴C in the total lipid extract of the blocks and the ratio of ³H to ¹⁴C in chylomicrons:

Chylomicron glyceride hydrolyzed (%)

$$= 100 \times \left(1 - \frac{{}^3\text{H}/{}^{14}\text{C in total lipid}}{{}^3\text{H}/{}^{14}\text{C in chylomicrons}} \right)$$

TABLE III
 Extraction of Palmitic Acid-¹⁴C during Processing of Plasma-Gelatin Blocks for Electron Microscopy

Plasma-gelatin blocks, fixed 30 min in 3% glutaraldehyde solution at 2°-4°C and washed 60-80 min in 0.1 M sodium cacodylate at 2°-4°C, were incubated at 38°C in 0.035 M CaCl₂-0.1 M Tris medium (pH 8.3) with or without 0.004 M E-600 (diethyl paranitrophenyl phosphate), treated with Pb(NO₃)₂-sodium cacodylate, postfixed in OsO₄, dehydrated with acetone, and infiltrated with Epon.

Group*	Incubation		No. of expts.	Present as FFA at end of incubation	Palmitic acid- ¹⁴ C (% of total ¹⁴ C)				Total extracted	Electron-lucent areas in chylomicrons [§]
	Addition	Time			hr	OsO ₄ -Caco	Acetone	Epon acetone		
I	None	2	3	4 ± 0.3	<0.1	1.5 ± 0.2	6.2 ± 1.1	<0.1	7.8 ± 1.1	None
II-A	None	0	3	35 ± 1.3	<0.1	5.7 ± 1.3	17.3 ± 1.3	<0.1	23.2 ± 1.3	None
II-B	E-600	2	3	36 ± 2.7	<0.1	4.8 ± 0.6	12.9 ± 3.6	<0.1	17.7 ± 4.1	Few
II-C	None	2	3	80 ± 1.0	<0.1	2.1 ± 0.3	30.0 ± 4.5	<0.1	32.0 ± 4.2	Many

* Group I, blocks prepared with chylomicrons and postheparin plasma mixed at 0°-2°C for 1 min.

Group II, blocks prepared with chylomicrons and postheparin plasma mixed at 24°C for 1 min. The chylomicrons contained triglyceride labeled with glycerol-³H and palmitic acid-¹⁴C.

† OsO₄-Caco = 2% OsO₄ in 0.1 M sodium cacodylate at 2°-4°C for 2 hr; acetone = 75-100% acetone at 4°C for 25 min; Epon acetone = 67% Epon in acetone at 24°C for 15 hr; and Epon = 100% Epon at 24°C for 8 hr.

§ Observed in electron-micrographs of blocks (Figs. 9-12).

RESULTS

Morphology of Chylomicrons

The structure of rat chylomicrons fixed while suspended in albumin solution is shown in Fig. 1. The chylomicrons ranged in diameter from 300 to 6000 Å and contained a homogeneous core surrounded by a thin nonuniform surface coat which was dense at points of contact between chylomicrons. The suspension also contained small, extremely osmiophilic granular particles, with a subunit structure of 10-15 Å, that could be very low density lipoproteins of hepatic or intestinal origin.

Lipoprotein Lipase Activity in

Plasma-Gelatin Blocks

Plasma-gelatin blocks containing chylomicrons were used for studying hydrolysis of chylomicron-triglyceride by lipoprotein lipase under the same conditions as those employed for cytochemical study of lipase in fixed tissues (14, 39). The level of lipoprotein lipase activity was increased by using postheparin instead of normal control plasma (30). The plasma-gelatin blocks were fixed in glutaraldehyde and washed in cacodylate buffer before being incubated in the media described in Tables I-III. Blocks were taken at the beginning and at the end of incubations for biochemical and morphological studies.

BIOCHEMICAL STUDIES: Chylomicron glyceride was hydrolyzed to glycerol and FFA by lipoprotein lipase in glutaraldehyde-fixed gelatin blocks containing postheparin plasma when the blocks were incubated at 38°C at pH 8.3 (Tables I-II). The rate of hydrolysis was 100% higher in blocks incubated in CaCl_2 -Tris than in those incubated in Tris medium only, and lower in blocks incubated in $\text{Pb}(\text{NO}_3)_2$ -sodium cacodylate medium (Table II). The slower rate observed in the latter blocks could be due to the low pH (6.8) of 0.03 M $\text{Pb}(\text{NO}_3)_2$ -0.1 M sodium cacodylate medium, since the optimal pH for lipoprotein lipase activity is near 8.3 (19, 30). Hydrolysis of chylomicron glyceride in control plasma-gelatin blocks was very small, about 10% of that in blocks containing postheparin plasma (Table II). Hydrolysis was also less in blocks containing postheparin plasma if the chylomicrons and plasma had been mixed

together at 0°-2°C (group I) instead of at 24°C (group II-C) (Table III).

Hydrolysis of chylomicron triglyceride in plasma-gelatin blocks was decreased more than 95% by 0.004 M E-600, an inhibitor of lipoprotein lipase (20, 39) (Tables I and III). E-600, however, did not prevent the decrease in monoglyceride content, suggesting that it had little if any effect on the action of monoglyceride lipase.

The above findings show that chylomicron triglyceride is hydrolyzed to FFA and glycerol in incubated glutaraldehyde-fixed plasma-gelatin blocks containing lipoprotein lipase, and that hydrolysis is enhanced by Ca^{++} added to the incubation medium. Although hydrolysis occurred in the presence of Pb^{++} , the rate was less, probably because of the low pH needed to dissolve $\text{Pb}(\text{NO}_3)_2$ in cacodylate buffer. Both Ca^{++} and Pb^{++} react with FFA, long chain fatty acids, to form water-insoluble precipitates which can be detected by microscopy (13). Analyses of blocks and media showed that all of the FFA released from chylomicron glyceride were retained in the blocks.

MORPHOLOGICAL STUDIES: Chylomicrons in an unincubated gelatin block containing postheparin plasma are shown in Figs. 3 and 9. These chylomicrons, unlike those described in Fig. 1, did not have an electron-opaque surface coat, and they were often clustered together. They did have, however, a homogeneous core similar to that seen in chylomicrons fixed while in suspension. Studies elsewhere have shown that the core consists primarily of triglyceride (48, 49). There was no difference in appearance between chylomicrons in unincubated gelatin blocks containing postheparin plasma and those in blocks containing control plasma.

The effects of incubation on the appearance of chylomicrons in plasma-gelatin blocks are shown in Figs. 4-8 and 10-14. Chylomicrons embedded with control plasma showed practically no change in appearance after being incubated 1 hr in Tris buffer and treated with 0.06 M $\text{Pb}(\text{NO}_3)_2$ -0.1 M sodium cacodylate solution (Fig. 4), as would be anticipated from the biochemical finding that only 1% of the triglyceride had been hydrolyzed (Table II). A faint electron-opaque precipitate was deposited around the periphery of chylomicrons in control plasma-gelatin blocks when they were incubated in CaCl_2 -Tris medium and treated with Pb^{++} (Fig. 5). This probably re-

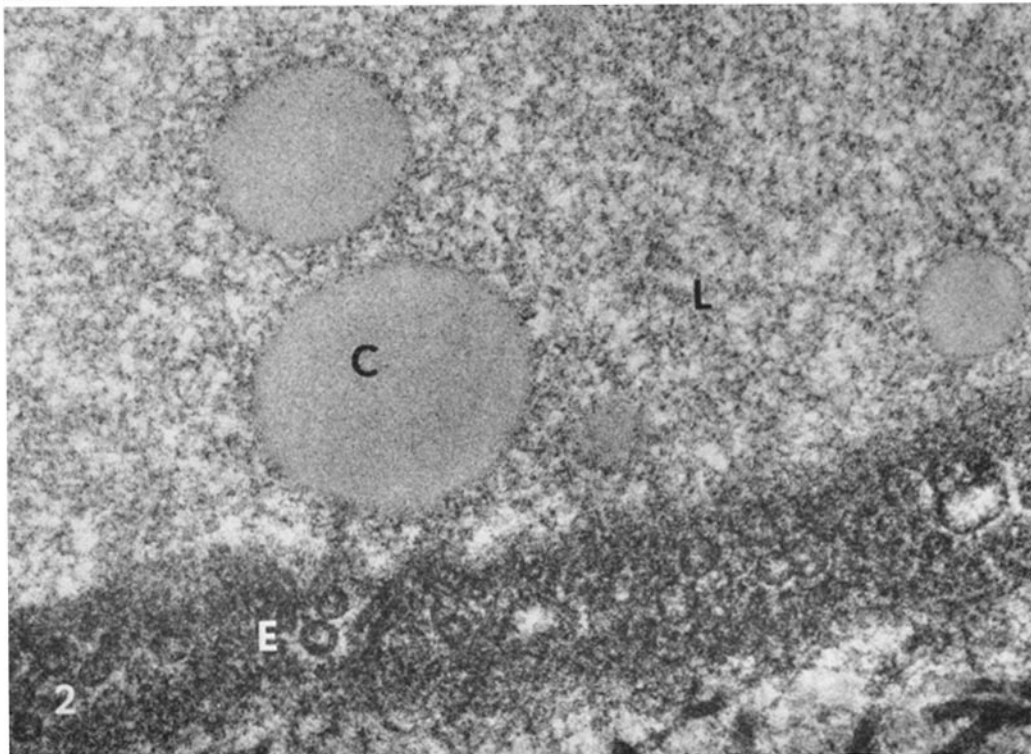
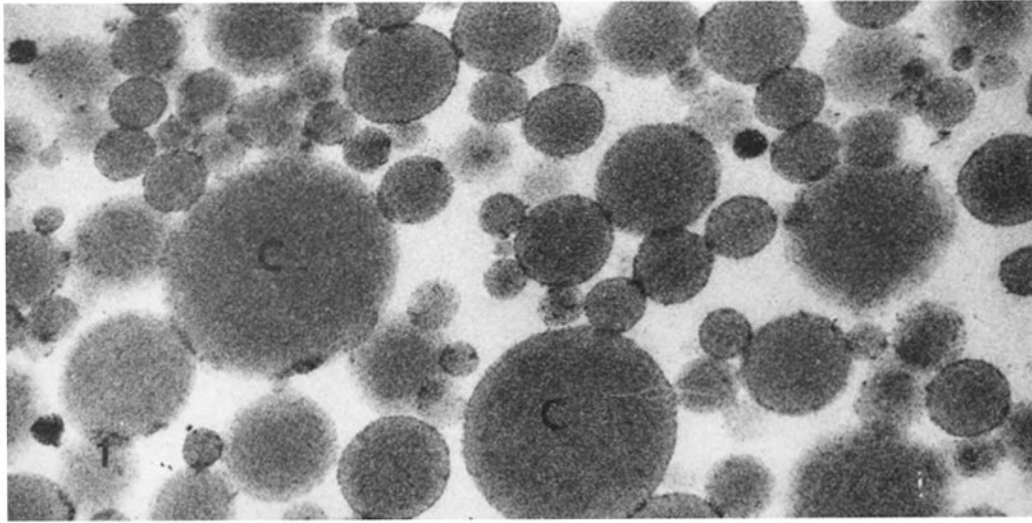


FIGURE 1 A section through chylomicrons (*C*) isolated from thoracic duct chyle of a rat fed corn oil, suspended in 4% albumin solution, and fixed with glutaraldehyde and osmium tetroxide (pH 7.4). Stained section. $\times 35,000$.

FIGURE 2 Chylomicrons in a capillary lumen (*L*) of a rat parametrial fat pad perfused 10 min with blood containing chylomicrons. Some of the chylomicrons (*C*) are present at the luminal surface of the endothelium (*E*). Stained section. $\times 70,000$.

flected trapping by the cations of the small amount of FFA released during incubation (Table II).

The appearance of chylomicrons was greatly affected when more of the triglyceride, 15–74%, was hydrolyzed (Figs. 6–8). There was loss of electron-opaque material from the core of chylomicrons (Figs. 6–8, 10 and 14) and deposition of granular (Figs. 6, 8, and 10) and laminar (Figs. 6, 13, and 14) electron-opaque precipitates within the electron-lucent areas and on the surface of the chylomicrons. The granular and laminar precipitates, however, were not seen after hydrolysis in blocks incubated in Tris or CaCl_2 -Tris medium unless the blocks were treated with Pb^{++} after incubation; electron-lucent areas, however, were present. The presence of laminar precipitates in blocks incubated in Tris medium and treated with Pb^{++} (Fig. 13) indicates that their formation was not dependent upon the presence of cations in the medium during the incubation. The laminar precipitates had a periodicity of 40–50 Å and appeared similar to polar lipid complexes observed in artificial preparations (21).

Electron-lucent areas and precipitates were not found in incubated blocks prepared with chylomicrons and plasma mixed at 0°–2°C (Fig. 11) instead of at 24°C. The chylomicrons, however, had an irregular contour (Fig. 11) in contrast to the regular contour of unincubated chylomicrons (Fig. 9). The irregular surface probably reflects the very small amount of hydrolysis that occurred during incubation, about 2% in 2 hr.

There was very little morphological evidence of hydrolysis in plasma-gelatin blocks incubated in CaCl_2 -Tris medium containing E-600, confirming the biochemical findings described above (Tables I and III). Some of the chylomicrons contained near their periphery electron-lucent areas outlined by granular electron-opaque precipitates (Fig. 12), whereas most of them had irregular contours (Fig. 12).

The possibility that electron-lucent areas in chylomicrons were due to extraction of lipid during the processing of blocks for electron microscopy was investigated by measuring the lipid- ^{14}C removed at each step. The results show that lipid was extracted from blocks by acetone and by Epon acetone, but not by OsO_4 -cacodylate solution or by 100% Epon, and that the amount extracted was related to the FFA content of the

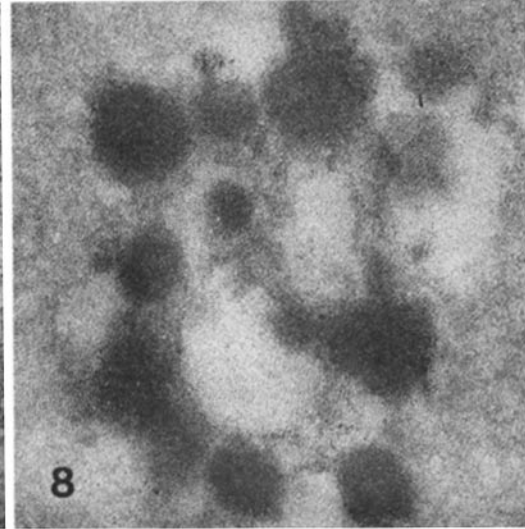
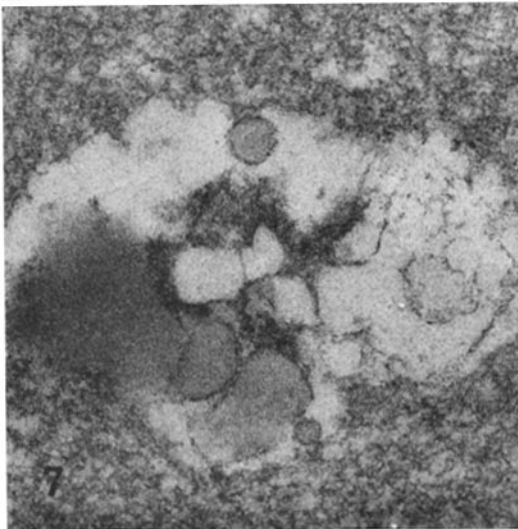
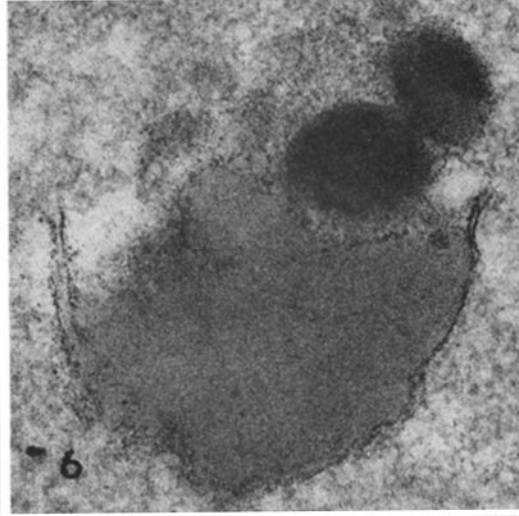
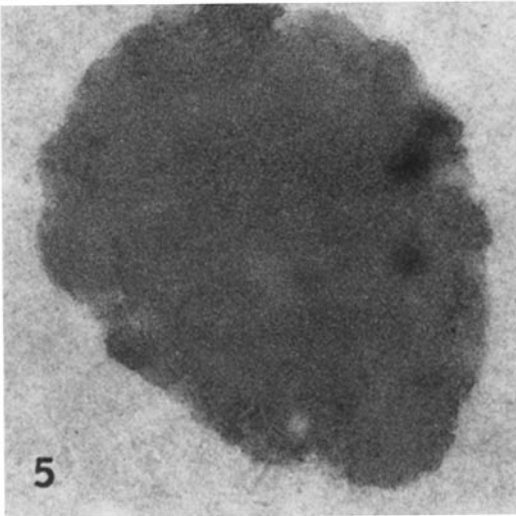
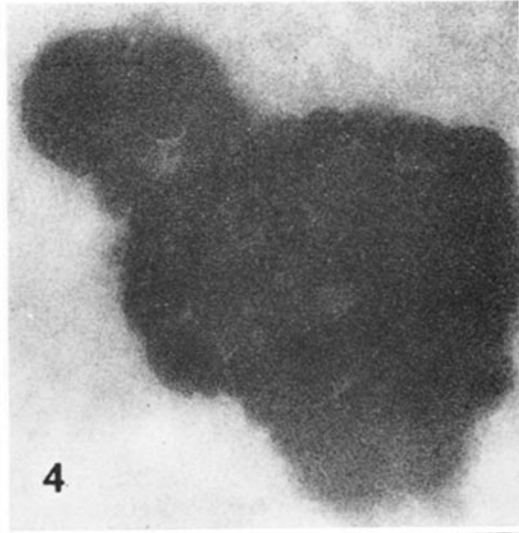
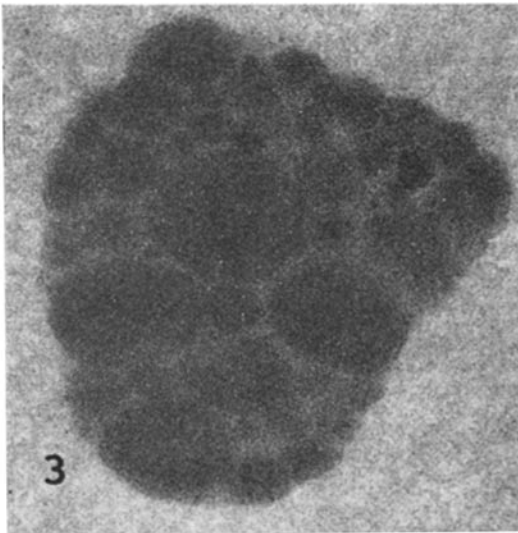
blocks (Table III). The findings in group I, which had 4% of the lipid present as FFA and 96% present as glyceride, indicate that no more than 8%, or less than 4%, of the glyceride was extracted. The amount of lipid extracted from blocks in the other groups however, exceeded 8%, indicating that FFA were also extracted. The differences between group I and the other groups suggest that at least $\frac{2}{3}$ of the lipid- ^{14}C extracted was FFA in group II-A, $\frac{1}{2}$ in group II-B, and $\frac{3}{4}$ in group II-C.

Electron micrographs showed no electron-lucent areas in chylomicrons in groups I (Fig. 11) and II-A (Fig. 9), a few in group II-B (Fig. 12), and many in group II-C (Fig. 10). The results in Table III show that the incidence of electron-lucent areas was not related to the amount of lipid hydrolyzed or to the amount extracted. Blocks in group II-A, which were unincubated, contained a large amount of FFA and lost $\frac{1}{5}$ of the lipid during processing but they did not have electron-lucent areas in chylomicrons. Since $\frac{3}{4}$ of the FFA present in these blocks were formed before fixation (Table I), it is likely that most of the FFA were bound to albumin (31, 42) and dispersed throughout the block before the proteins were fixed with glutaraldehyde. The effect of removing albumin-bound FFA, as well as formation of lead salts with albumin-bound FFA, probably would be too diffuse to be detected in electron micrographs. Since half of the FFA in group II-C was released from glyceride after fixation, it is possible that the electron-lucent areas in chylomicrons of this group were caused during processing by extraction of fatty acid that had been released during incubation and deposited as soaps at or near sites of hydrolysis. The smaller number and size of electron-lucent areas in group II-B (Table III) (Fig. 12) reflect decreased hydrolysis during incubation in the presence of E-600 (Table I).

These studies show that hydrolysis of chylomicron glyceride by lipoprotein lipase in glutaraldehyde-fixed plasma-gelatin blocks can be demonstrated cytochemically by loss of electron opacity in chylomicrons and by deposition in or near chylomicrons of granular and laminar electron-opaque precipitates.

Structure of Capillaries in Adipose Tissue

The capillaries of parametrial adipose tissue were lined by a continuous endothelium sur-



rounded by a basement membrane and many pericytes (Fig. 15). Although interendothelial junctional complexes were present, sometimes the space between endothelial cells, about 200 Å wide, extended from the lumen to the basement membrane. There were numerous microvesicles (300 Å) and many-sized vacuoles in the endothelial cells. Some of the vacuoles extended from the luminal to the basal surface of the cell (Fig. 16), thus decreasing the structural barrier between capillary lumen and subendothelial space. Although the margins of such vacuoles appeared indistinct, they could be membranous in structure (Fig. 16, arrows). The pericytes underlying the endothelium were enveloped by the capillary basement membrane (Figs. 15, 16, 20, 25, and 28).

Adipose tissue perfused with chylomicrons contained intact chylomicrons dispersed throughout the capillary lumen (Figs. 2, 15, and 24). The chylomicrons were similar in size, shape, and electron opacity (Figs. 2, 15, and 17) to those fixed in suspension (Fig. 1), but they did not have an electron-opaque surface coat (Fig. 2). Chylomicrons were also seen adhered to (Fig. 19)

or partially enveloped by endothelial cells (Figs. 15, 17, and 18). The enveloped chylomicrons were in direct contact with microvesicular invaginations of the endothelial plasma membrane (Fig. 17). Microvesicles were also contiguous with vacuoles that contained (flocculent) material similar to that in chylomicrons (Figs. 17 and 18). Many microvesicles were present at the luminal, basal, and lateral surfaces of endothelial cells (Figs. 2, 19, 21, and 22). Vacuoles were also numerous in endothelial cells of tissue perfused with chylomicrons (Fig. 15).

Lipoprotein Lipase Activity in Adipose Tissue Perfused with Chylomicrons

Parametrial fat pads perfused with doubly labeled chylomicrons were used to study, biochemically and cytochemically, lipoprotein lipase activity in adipose tissue. The perfusion was stopped while chylomicrons were flowing through the tissue, and the tissue was immediately chilled to $< 4^{\circ}\text{C}$ and fixed in ice-cold glutaraldehyde solution. The tissues were then incubated in CaCl_2 -Tris medium (pH 8.3) and analyzed bio-

FIGURES 3-15 Chylomicrons in plasma-gelatin blocks containing either control or postheparin plasma.

FIGURE 3 Postheparin plasma. A section through an aggregation of chylomicrons in an unincubated plasma-gelatin block. Unstained section. $\times 45,000$.

FIGURE 4 Control plasma. The block was incubated 1 hr in Tris medium (pH 8.3) and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. 1% of the chylomicrons were hydrolyzed during incubation (Table II). The chylomicrons are intact and exhibit a homogeneous osmiophilia similar to that in unincubated gelatin blocks (Fig. 3). Unstained section. $\times 45,000$.

FIGURE 5 Control plasma. The block was incubated 1 hr in CaCl_2 -Tris medium (pH 8.3) and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. 6% of the chylomicrons were hydrolyzed during incubation (Table II). Note homogeneity of osmiophilia and very slight electron-opaque precipitate at the periphery of some of the chylomicrons. Unstained section. $\times 61,000$.

FIGURE 6 Postheparin plasma. The block was incubated 1 hr in $\text{Pb}(\text{NO}_3)_2$ -cacodylate medium (pH 6.8). 15% of the chylomicrons were hydrolyzed during incubation (Table II). Laminar and granular electron-opaque precipitates are present at the periphery of chylomicrons and in the electron-lucent areas. Unstained section. $\times 87,000$.

FIGURE 7 Postheparin plasma. The block was incubated 1 hr in CaCl_2 -Tris medium, and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. Biochemical studies showed that 57% of the chylomicrons were hydrolyzed (Table II). The large electron-lucent areas also indicate that a large proportion were hydrolyzed. Electron-opaque deposits are present at the periphery of chylomicrons and in the electron-lucent areas. Unstained section. $\times 48,000$.

FIGURE 8 Postheparin plasma. The block was incubated 3 hr in Tris medium and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. 74% of the chylomicrons were hydrolyzed (Table II). The large electron-lucent areas resulted from hydrolysis of chylomicrons. Some of the chylomicrons are circumscribed by lead precipitates. Unstained section. $\times 95,000$.

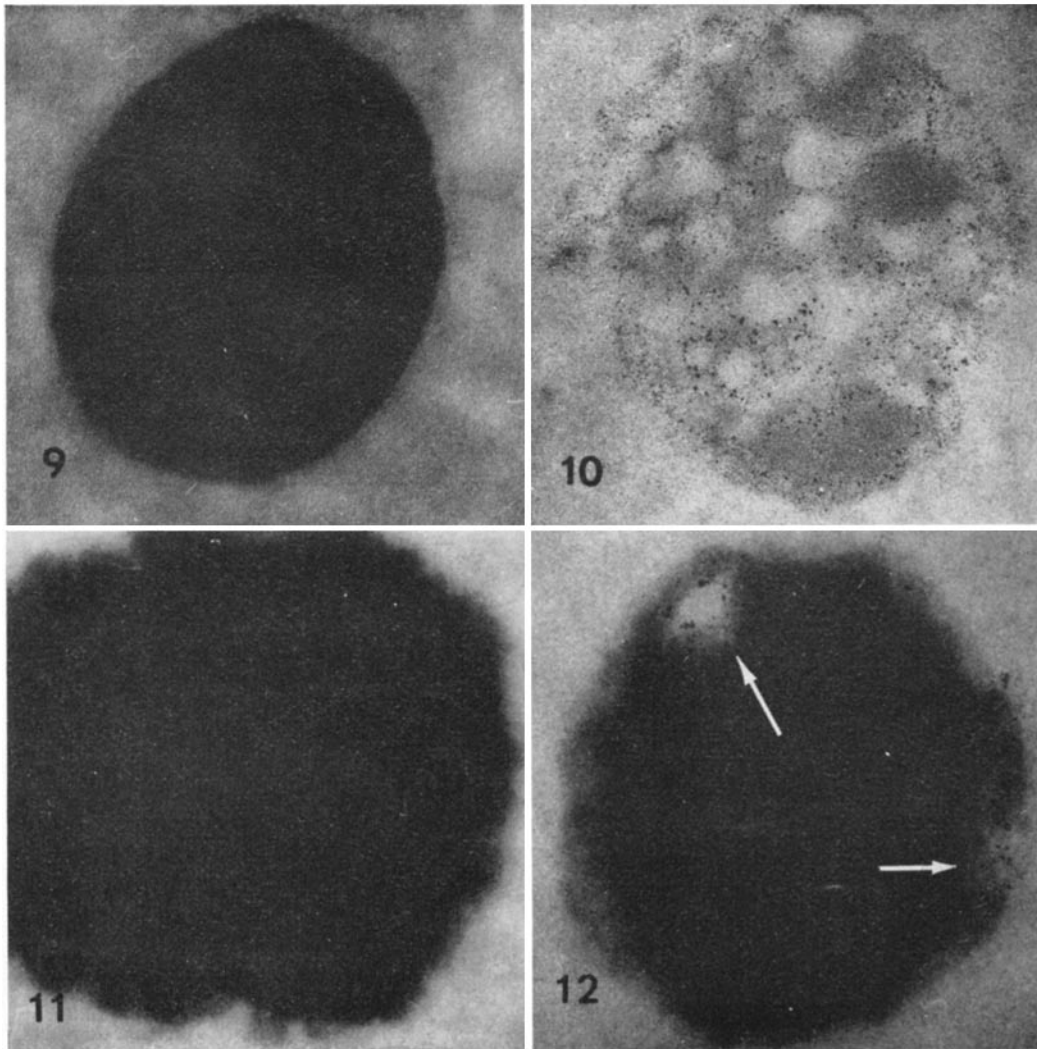


FIGURE 9 Postheparin plasma. A section through an unincubated plasma-gelatin block (group II-A, Table III). The chylomicron has a homogeneous core and a regular surface. Unstained section. $\times 115,000$.

FIGURE 10 Postheparin plasma. The block was incubated 2 hr in CaCl_2 -Tris medium and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution (group II-C, Table III). The large electron-lucent areas containing electron-opaque precipitate indicate that a large proportion of the chylomicrons were hydrolyzed. The electron-lucent areas were probably produced during processing by the extraction of fatty acid soaps formed during incubation. Unstained section. $\times 61,000$.

FIGURE 11 Postheparin plasma. The chylomicrons and plasma were mixed for 1 min at 0° - 2°C , instead of at 24°C , before they were added to gelatin solution. The block was incubated 1 hr in CaCl_2 -Tris medium and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution (group I, Table III). The chylomicron has a homogeneous core, but the surface is irregular, probably due to the small amount of hydrolysis that occurred during incubation. Unstained section. $\times 112,000$.

FIGURE 12 Postheparin plasma. The block was incubated 2 hr in CaCl_2 -Tris medium containing 0.004 M E-600 and then treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution (group II-B, Table III). Note the small discrete electron-lucent areas (arrows) outlined by electron-opaque precipitate at the periphery of the chylomicron. Compare with Fig. 10. Unstained section. $\times 86,000$.

chemically (Tables IV and V) and morphologically (Figs. 19-28).

BIOCHEMICAL STUDIES: During the perfusion parametrial fat pads hydrolyzed chylomicron triglyceride to FFA and incorporated the FFA into tissue triglyceride (Table IV). Both processes continued after the perfusion was stopped if the temperature was maintained at 38°C; 65% of the chylomicron triglyceride remaining in the tissue was hydrolyzed in 15 min and the FFA released were reesterified (Table IV). Further hydrolysis did not occur when incubation was prolonged another 15 min.

Preliminary experiments showed that hydrolysis occurred in tissue during fixation if the temperature was not reduced. Chilling and fixing at < 4°C, however, stopped both hydrolysis and reesterification (Table IV). Hydrolysis, but not reesterification, resumed when the tissues were incubated at 38°C in CaCl₂-Tris medium (Tables IV and V).

E-600, at a concentration of 0.004 M, decreased hydrolysis of chylomicron glyceride by 80% in fixed tissues incubated at 38°C (Table V).

MORPHOLOGICAL STUDIES: Hydrolysis of chylomicron triglyceride occurred during incu-

bation in the lumen of capillaries of tissues that were not chilled and fixed immediately after perfusion (Fig. 26). The lumina contained highly organized, laminar electron-opaque deposits that were similar to those seen in incubated post-heparin plasma-gelatin blocks (Figs. 13 and 14). The laminations of the deposits in both preparations had a periodicity of 40-50 Å.

Hydrolysis did not occur during incubation in capillary lumina of tissues chilled and fixed immediately after perfusion; the chylomicrons remained unchanged and electron-opaque (Figs. 19, 23, and 24). Evidence of hydrolysis, however, was present in the endothelium and in the sub-endothelial space. Laminar deposits were seen in intra-endothelial vacuoles (Figs. 19-21, 23, and 24) and in the space between the pericyte and endothelial cell (Figs. 20 and 23-26), and sometimes immediately beyond the pericyte. Discrete aggregates of finely particulate, electron-opaque precipitates were present in apical and basal microvesicles (Fig. 22), and sometimes in lateral microvesicles, of endothelial cells; these precipitates were visible in unstained sections. There was no evidence of hydrolysis within fat cells or

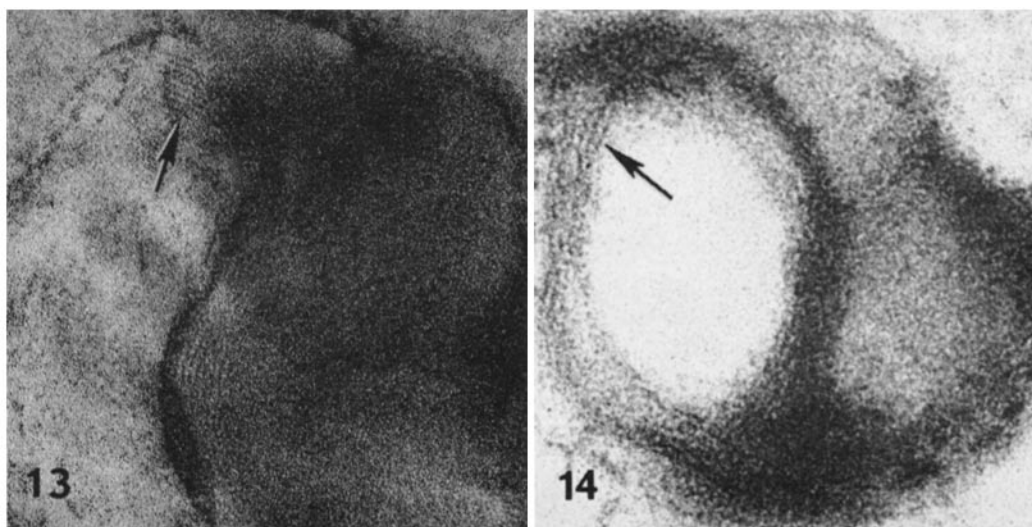
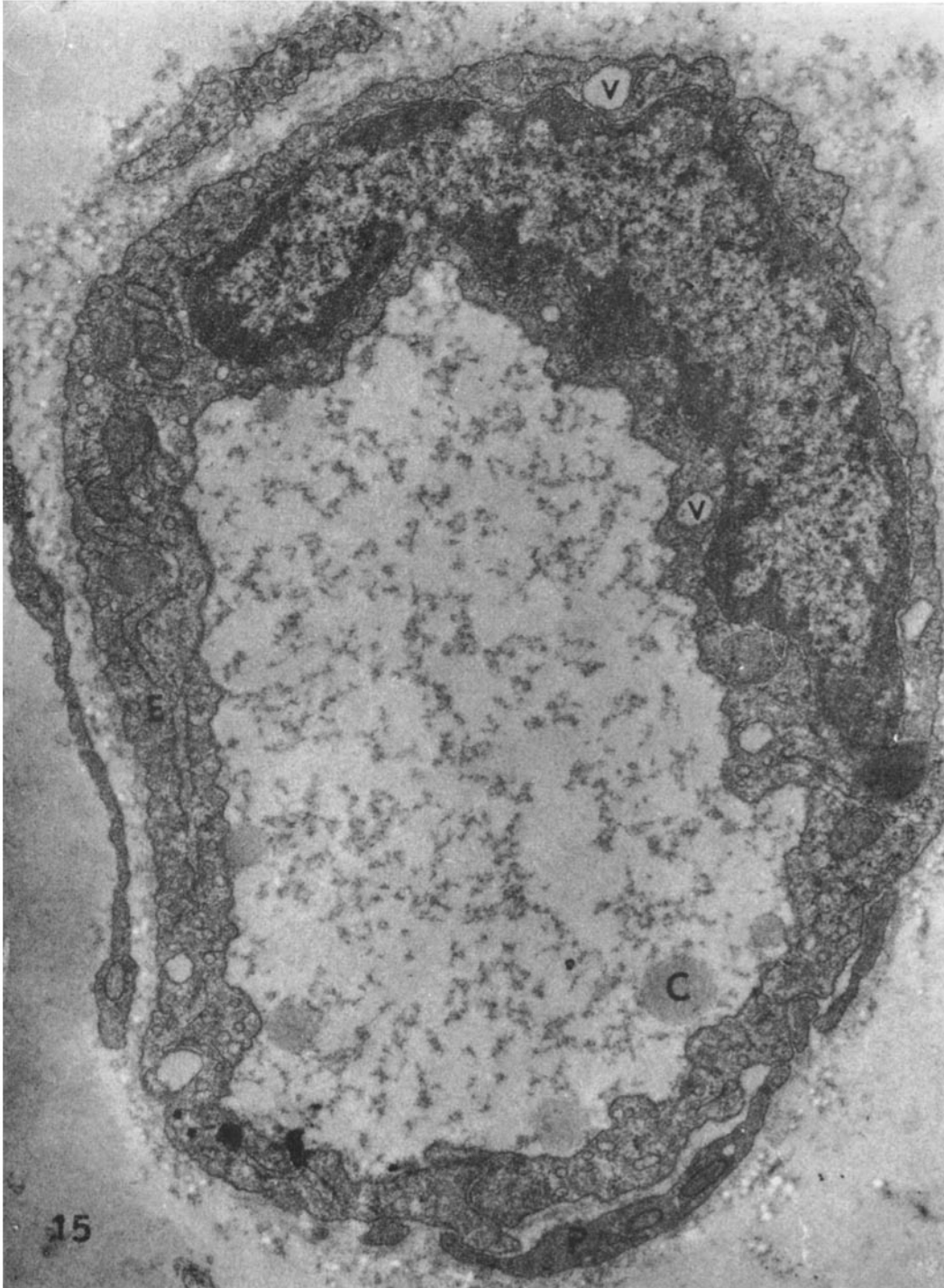


FIGURE 13 Postheparin plasma. The block was incubated 1 hr in Tris medium and treated 15 min with Pb(NO₃)₂-cacodylate solution. The laminar electron-opaque structures (arrow) in and near the electron-lucent area have a periodicity of 40-50 Å. Unstained section. × 151,000.

FIGURE 14 Postheparin plasma. The block was incubated 1 hr in CaCl₂-Tris medium and treated 15 min with Pb(NO₃)₂-cacodylate solution. Laminar electron-opaque structures with 40-50 Å spacing (arrow) surround the electron-lucent area. Stained section. × 240,000.



15

FIGURE 15 Capillary of a rat parametrial fat pad perfused 10 min with blood containing chylomicrons. Chylomicrons (*C*) are present in the lumen near the endothelium (*E*). Note the large vacuoles (*V*) inside and the pericytes (*P*) surrounding the endothelium. Stained section. $\times 23,000$.

in the immediate extravascular space surrounding fat cells.

Apparent swelling occurred in the vicinity of the larger aggregates of precipitates in the sub-endothelial space (Figs. 24 and 25). This phe-

nomenon, which may have resulted from local change in osmolality or pH during hydrolysis, was not seen in unincubated perfused or in incubated unperfused tissue.

Prolonging the incubation of tissue blocks from

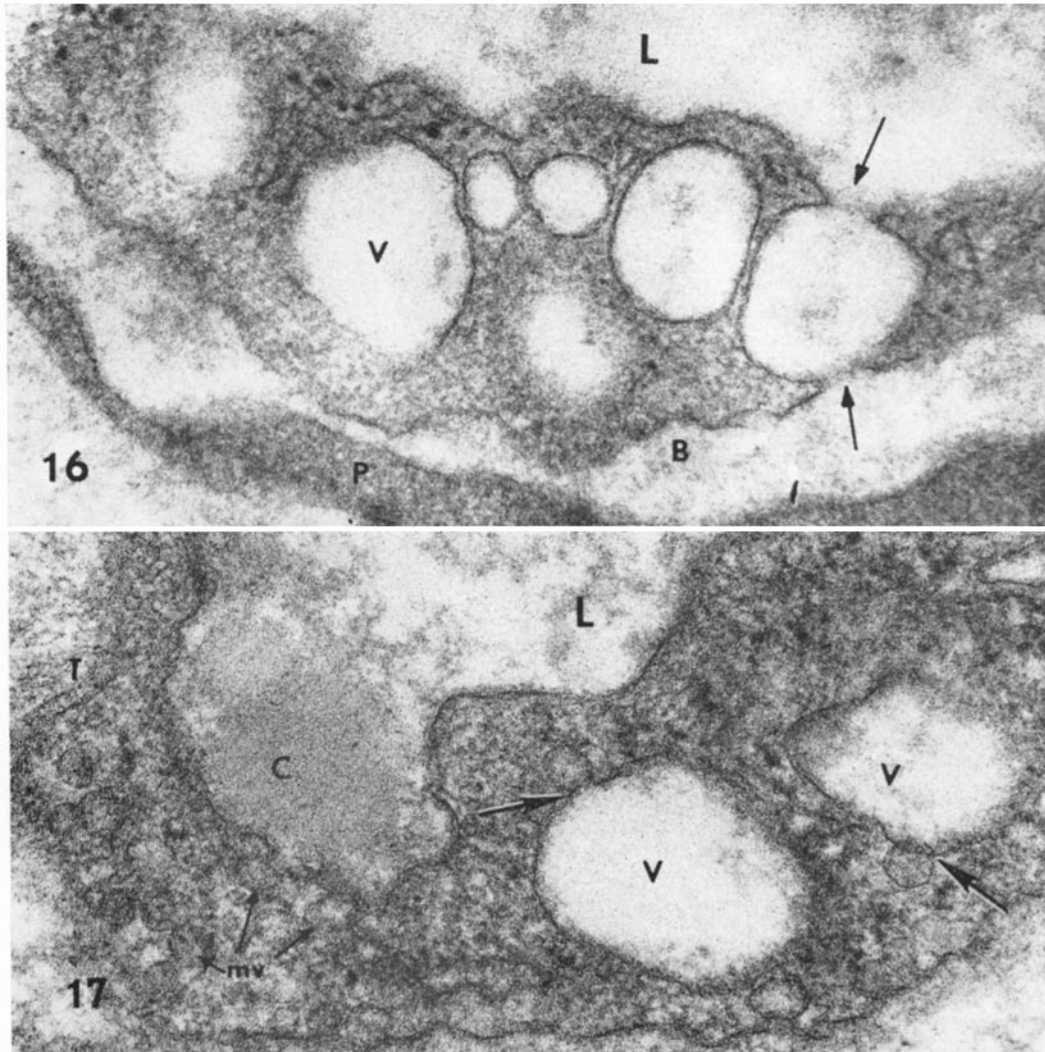


FIGURE 16 Detail of a capillary endothelium in an unperfused fat pad. Numerous vacuoles (V) approximately 0.25μ in diameter are present in the cytoplasm. The surface of one vacuole is so attenuated that very fine osmiophilic densities (arrows) appear to be the only structural barrier between the lumen (L) and the subendothelial space (B). Pericyte, P. Stained section. $\times 99,000$.

FIGURE 17 Detail of a capillary endothelium in a parametrial fat pad perfused 5 min with chylomicrons. A chylomicron (C) partially enveloped by an endothelial cell shows peripheral dispersion at the sites of microvesicular invagination (mv). Note the junctions (arrows) between microvesicles and vacuoles (V). Both microvesicles and vacuoles contain flocculent material similar in electron opacity to that in the chylomicron. A tubular profile (T) 300 A in diameter is evident in the cytoplasm. Stained section. $\times 89,000$.

1 hr to 3 hr produced no consistent cytochemical change at the fine structural level. Cytochemical studies were also made in sections cut at 30–50 μ with a cryostat after fixation of the tissue. The results obtained in sections incubated 0.5–1 hr were similar to those obtained in tissue blocks. However, this technique was not used routinely because the fat cells were frequently distorted in the sections.

E-600 added to the incubation medium decreased markedly the amount of laminar and granular electron-opaque precipitate deposited in the endothelium and subendothelial space of perfused tissue. An amorphous mass outlined by laminar electron-opaque precipitate was found between a fat cell and an elongated connective tissue cell, perhaps a pericyte, near a capillary of tissue incubated with E-600 (Fig. 28). The electron opacity of the mass was similar to that of the lipid droplet within the fat cell.

Granular and laminar electron-opaque precipitates were not found in incubated unperfused or in unincubated perfused tissue treated with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution.

DISCUSSION

Sites of lipoprotein lipase activity in adipose tissue during uptake of chylomicrons from blood were determined in the present study with a cytochemical technique (39) based on Gomori's histochemical method for lipase (13, 14). In most cytochemical procedures, substrate for the reaction being studied is added after the tissue is fixed. In the present study, however, the tissue

was first perfused with blood containing the substrate and then fixed. This allowed the substrate, chylomicron triglyceride, and the enzyme, lipoprotein lipase, to combine naturally. The enzymic reaction was arrested and restricted to its physiological site by chilling ($< 4^\circ\text{C}$) and fixing the tissue immediately after the perfusion was stopped. The reaction in fixed tissue was reactivated by incubating at 38°C , and the fatty acids released formed within the tissue insoluble electron-opaque precipitates. The pH of the incubation medium was 8.3, which is optimal for lipoprotein lipase activity (19, 30).

The nature of the products formed during the cytochemical procedure was studied in plasma-gelatin blocks fixed with glutaraldehyde. Biochemical studies showed that chylomicron triglyceride was hydrolyzed by lipoprotein lipase to FFA and glycerol and that the rate was increased by adding Ca^{++} to the medium. Morphological studies showed that chylomicrons became less electron-opaque when they were hydrolyzed, and that granular and laminar electron-opaque precipitates, formed by the reaction of Pb^{++} with insoluble fatty acid soaps, were deposited at the sites of hydrolysis. The electron-lucent areas in the chylomicrons were probably caused during processing of the blocks by extraction of fatty acid soaps formed during incubation. Laminar and granular electron-opaque precipitates, and occasionally electron-lucent areas, were found after incubation in chylomicron-perfused but not in unperfused adipose tissue. Biochemical and morphological evidence of hydrolysis of chylo

FIGURE 18 Detail of a capillary endothelium in a parametrial fat pad perfused 5 min with chylomicrons. Note the chylomicron (*C*) near the luminal border and the flocculent material present at the invaginations of the plasma membrane (arrow). Microvesicles (*mv*) and a large vacuole (*V*) appear to be continuous with the invaginations. The vacuole contains osmiophilic material similar to that seen in chylomicrons. Stained section. $\times 91,000$.

FIGURE 19 Cytochemical reaction in a capillary of a fat pad perfused 5 min with chylomicrons, incubated 1 hr in CaCl_2 -Tris medium, and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. An intact chylomicron (*C*) is present in the capillary lumen (*L*). Note laminar deposit (*d*) within a vacuole and granular precipitate outlining a circular profile within the cell. The laminar and granular precipitates indicate sites of hydrolysis of glyceride. Unstained section. $\times 83,000$.

FIGURE 20 Cytochemical reaction in a capillary of a fat pad prepared as in Fig. 19. Note the circular profiles (*d*) within the endothelial cell and in the subendothelial space. These profiles indicate that glyceride was hydrolyzed within the endothelium and in the space between the endothelium and pericyte (*P*). Capillary lumen, *L*. Unstained section. $\times 80,000$.

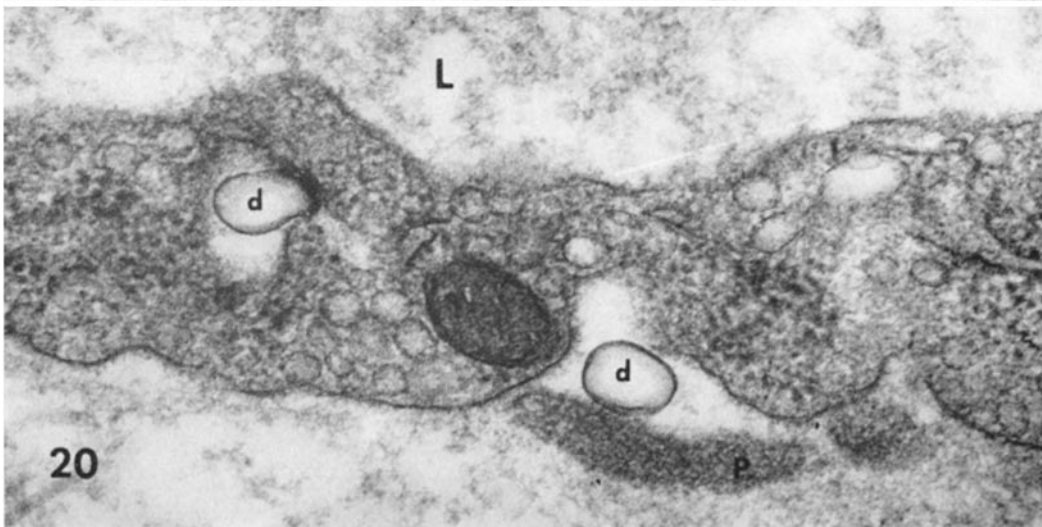
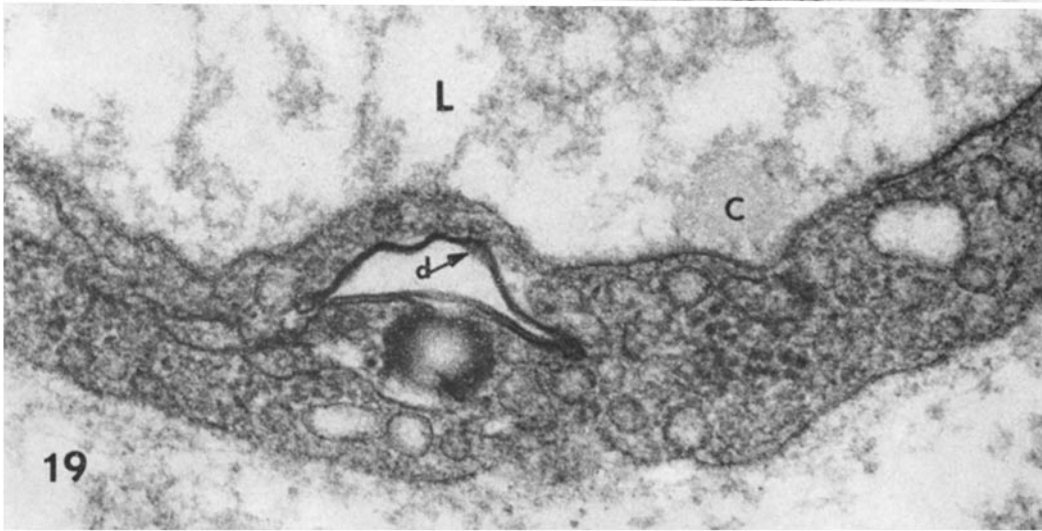
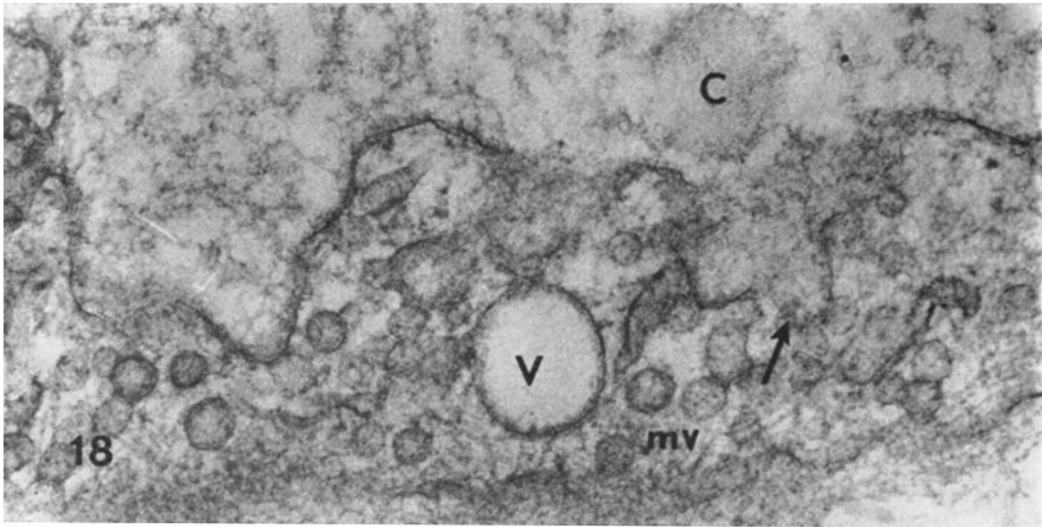


TABLE IV

Effect of Glutaraldehyde-Fixation on Hydrolysis and Reesterification of Chylomicron-Triglyceride during Incubation of Parametrial Adipose Tissue Perfused with Doubly Labeled Chylomicrons

Parametrial fat pads were perfused 4-5 min with blood containing doubly labeled chylomicrons (glyceride-glycerol-³H and palmitic acid-¹⁴C) and immediately put into cacodylate buffer (pH 7.4) at <4°C. The distal portion was then cut into six pieces: one piece was put at once into chloroform:methanol (unfixed, 0 time), two pieces were incubated at once (unfixed), and three were fixed 15 min in glutaraldehyde at <4°C and washed 30 min in cacodylate buffer at <4°C before being incubated. The tissues were incubated at 38°C in CaCl₂-Tris medium (pH 8.3). The values are means of three experiments.

Distribution of palmitic acid- ¹⁴ C						
Incuba- tion time	Unfixed tissue			Fixed tissue		
	Chylo- microns	New TG	FFA	Chylo- microns	New TG	FFA
hr	% total			% total		
0	71	24	5	70	25	5
1/4	24*	69*	7	—	—	—
1/2	23*	72*	5	—	—	—
1	—	—	—	63	27	10
2	—	—	—	64	24	12

* Indicates values significantly different from that at 0 time ($P < 0.05$).

micron was markedly decreased when blocks and tissues were incubated with E-600, an inhibitor of lipoprotein lipase (20, 39). It is concluded that the laminar and granular electron-opaque precipitates, in both plasma-gelatin blocks and in tissues, indicate sites of hydrolysis of chylomicron glyceride by lipoprotein lipase.

The present study provides cytochemical evidence that hydrolysis of chylomicron triglyceride in adipose tissue occurs in capillary endothelial cells and in the subendothelial space, i.e., between the endothelium and pericytes (Figs. 20-25). Hydrolysis may also occur within chylomicrons adhered to and entrapped by the endothelium (Fig. 25), but not in free-flowing chylomicrons (Figs. 19, 23, and 24). Intraluminal hydrolysis of chylomicrons during incubation was observed, but only in tissues that were not chilled and fixed immediately after perfusion (Fig. 26); the amount of chylomicron triglyceride hydrolyzed in these tis-

TABLE V

Effect of E-600 on Hydrolysis of Chylomicron-Triglyceride during Incubation of Adipose Tissue Perfused with Doubly Labeled Chylomicrons

Parametrial fat pads were perfused 5 min with blood containing doubly labeled chylomicrons (glyceride-glycerol-³H and palmitic acid-¹⁴C), fixed 30 min in glutaraldehyde solution at 2°-4°C, washed 60 min in cacodylate solution at 2°-4°C, and then incubated at 38°C in CaCl₂-Tris medium (pH 8.3) with or without E-600 (0.004 M).

Addition	Incubation Time	Distribution of palmitic acid- ¹⁴ C*		
		Chylomicrons	New TG	FFA
	hr	% total	% total	% total
None	0	56 ± 4	36 ± 4	8 ± 1
None	1	46 ± 5‡	36 ± 4	18 ± 3‡
E-600	1	54 ± 3	35 ± 4	11 ± 2

* Mean ± SE of eight experiments.

‡ Values significantly different from that at 0 time ($P < 0.005$).

sues was also increased. This finding is considered to be an artifact caused by abnormal release of lipoprotein lipase to the capillary lumen during the prolonged interval between stopping the blood flow, and chilling and fixing the tissue. The conclusion that intraluminal hydrolysis is an artifact in these tissues is based on the following observations. Intraluminal hydrolysis during incubation was not seen in tissues chilled (< 4°C) and fixed immediately after perfusion (Figs. 19, 23, and 24), and lipoprotein lipase activity in venous blood of adipose tissue is extremely low unless heparin is added to the arterial blood (16, 35).

Intact chylomicrons in perfused adipose tissue were present primarily in the lumen of the capillary and to a lesser extent partially enveloped by the luminal surface of the endothelium (Figs. 2, 17, 19, and 24). Flocculent material similar to that in chylomicrons was seen in vacuoles within endothelial cells (Fig. 17). There was no direct morphological evidence that glyceride was present within microvesicles, in the subendothelial space, or in the extravascular space, confirming earlier reports (46, 47). However, the cytochemical studies provide evidence that glycerides were present at the end of the perfusion in vacuoles and microvesicles of the capillary endothelium and in the subendothelial space; these glycerides were hydrolyzed during incubation and the resultant fatty

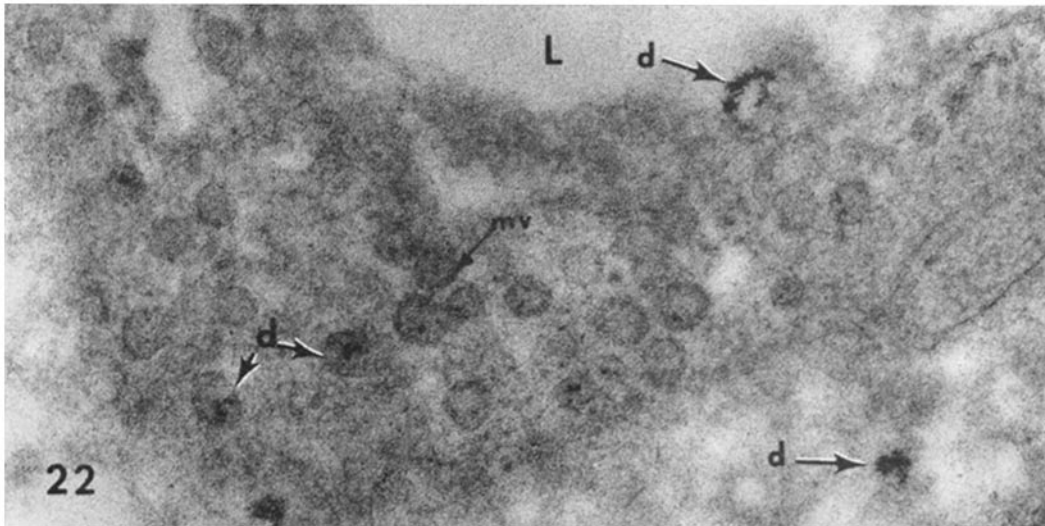
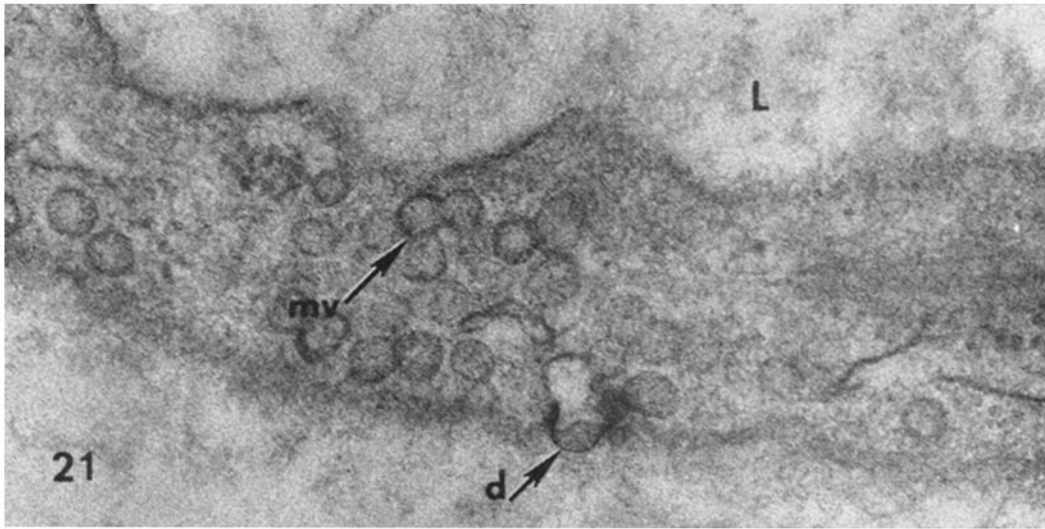


FIGURE 21 Cytochemical reaction in a capillary of a fat pad prepared as in Fig. 19. Note the laminar deposit (*d*) in continuity with a microvesicle (*mv*) at the basal surface of the endothelial cell. Capillary lumen, *L*. Stained section. $\times 96,000$.

FIGURE 22 Cytochemical reaction in a capillary endothelial cell of a fat pad perfused 5 min with chylomicrons, incubated 3 hr in CaCl_2 -Tris medium, and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. A dense reaction product outlines a circular profile (*d*) at the luminal border. Discrete deposits (*d*) are present also within microvesicles and in the subendothelial space. Capillary lumen, *L*; microvesicles, *mv*. Unstained section. $\times 102,000$.

acid soaps formed electron-opaque precipitates with Pb^{++} . An amorphous mass having characteristics of glyceride was found in the extracellular space of tissue incubated with lipase inhibitor (E-600); it was outlined by laminar precipitate and was similar in electron opacity to that of triglyc-

eride in the adipose cell (Fig. 28). The chemical nature of glycerides in the endothelium and extracellular space is not known.

Uptake of chylomicrons from blood by adipose tissue has been studied with electron microscope radioautography in perfused fat pads of starved

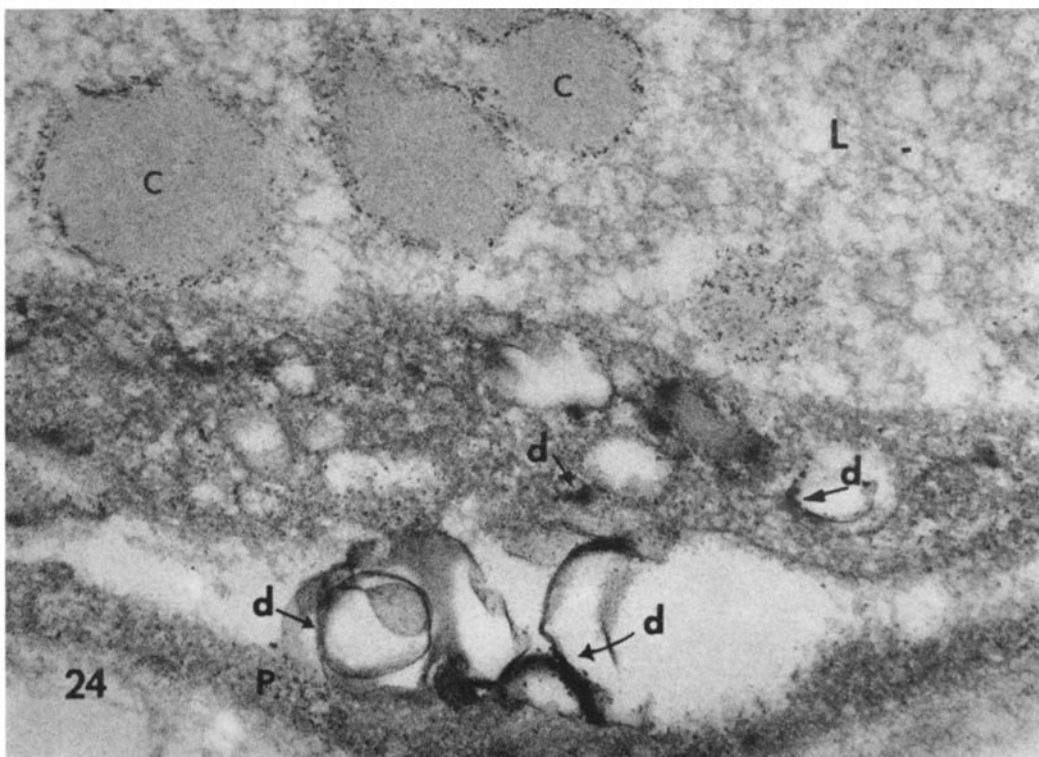
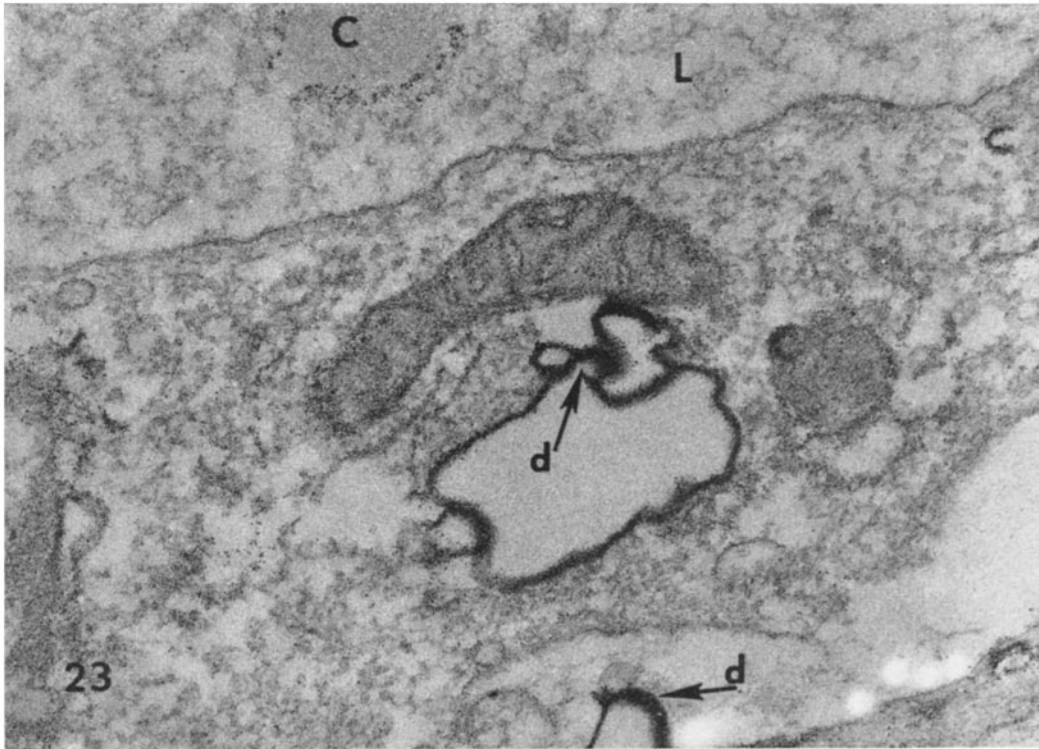


FIGURE 23 Cytochemical reaction in a capillary of a fat pad prepared as in Fig. 19. The chylomicron (*C*) in the lumen (*L*) exhibits homogeneous density and a slight irregularity of the surface (compare with Fig. 2). Intra-endothelial vacuoles contain laminar electron-opaque deposits (*d*). Electron-opaque deposits (*d*) are also present in the subendothelial area. Stained section. $\times 67,000$.

FIGURE 24 Cytochemical reaction in a capillary of a fat pad prepared as in Fig. 19. Intact chylomicrons (*C*) showing little evidence of hydrolysis are present in the lumen (*L*). Reaction products (*d*) depicting sites of hydrolysis of chylomicron glyceride are evident in intra-endothelial vesicles and in the subendothelial space between the endothelium and pericyte (*P*). Stained section. $\times 55,000$.

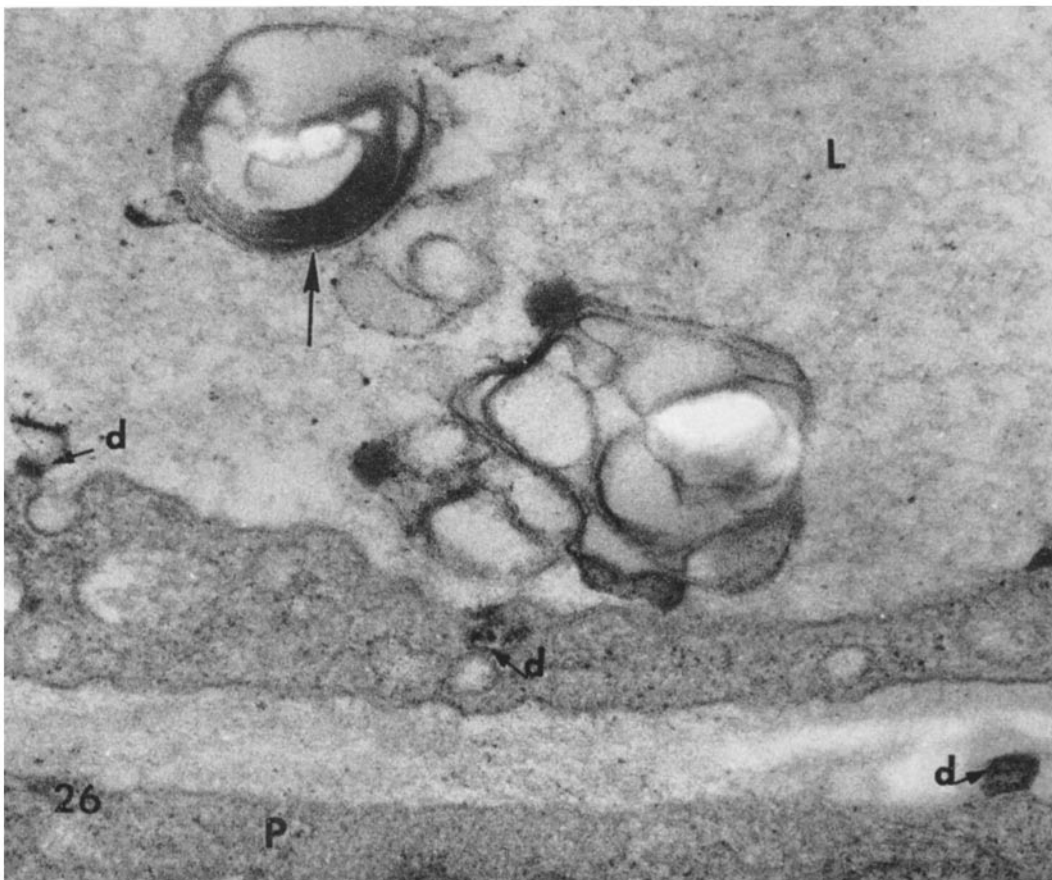
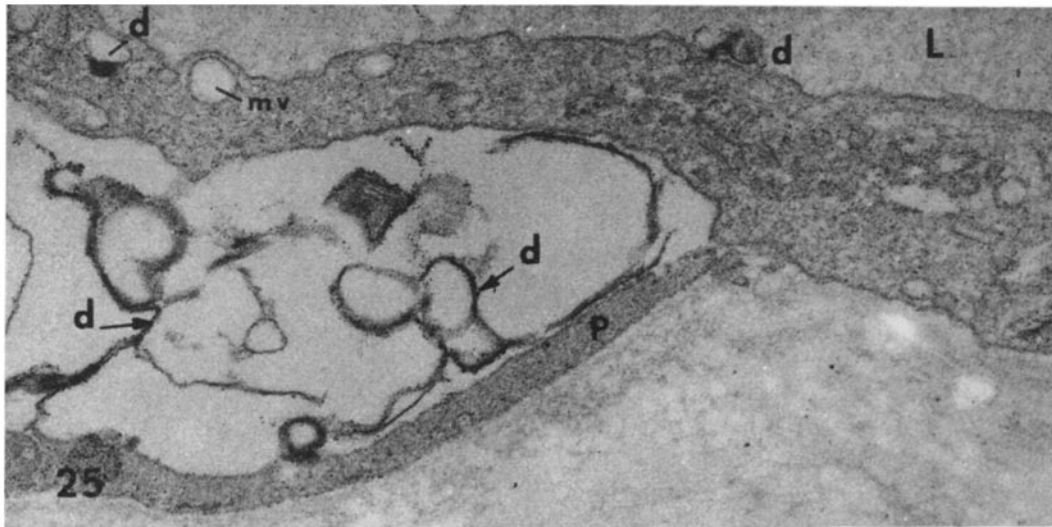


FIGURE 25 Cytochemical reaction in a capillary of a fat pad perfused 10 min with chylomicrons, incubated 3 hr in CaCl_2 -Tris medium, and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. Microvesicles (*mv*) approximately 1000 Å in diameter are present at the luminal border. Irregular profiles of electron-opaque material (*d*) are present at the luminal surface and within a cytoplasmic vesicle. There is a large amount of reaction product (*d*) in the space between the capillary and the pericyte (*P*). Stained section. $\times 55,000$.

FIGURE 26 Cytochemical reaction in a capillary of a fat pad perfused 10 min with chylomicrons, fixed in glutaraldehyde *without* chilling below 5°C , incubated 3 hr in CaCl_2 -Tris medium, and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. The large intraluminal masses represent products formed by the reaction of FFA with Ca^{++} and Pb^{++} . The organized laminations (arrow) should be compared with those in Figs. 13 and 14. Small electron-opaque deposits (*d*) are present in microvesicles and in the subendothelial space. Pericyte, *P*. Stained section. $\times 80,300$.

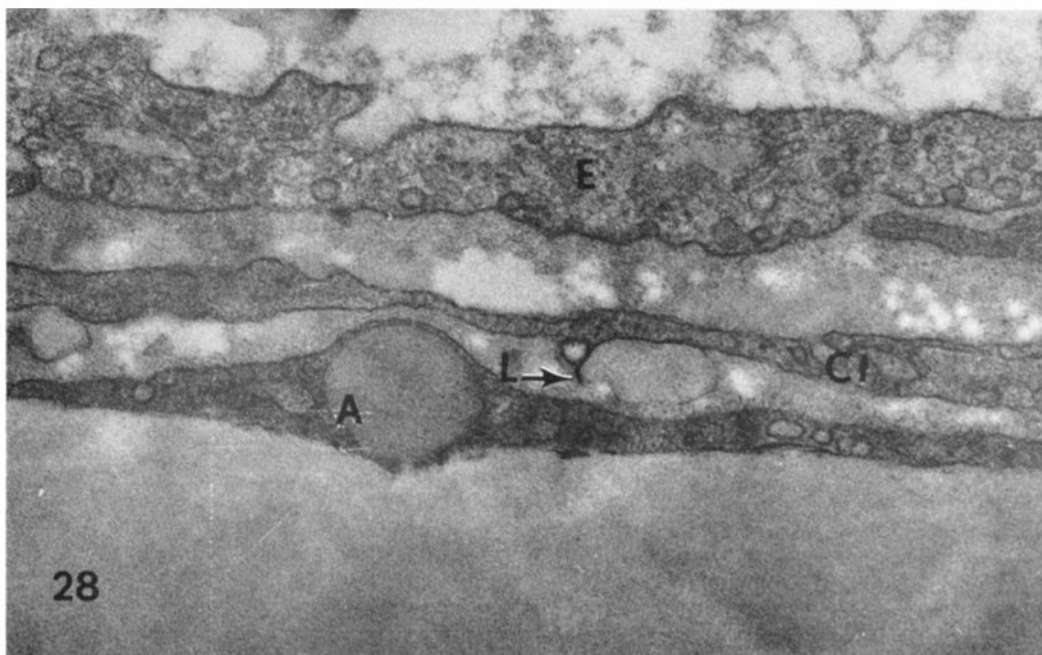
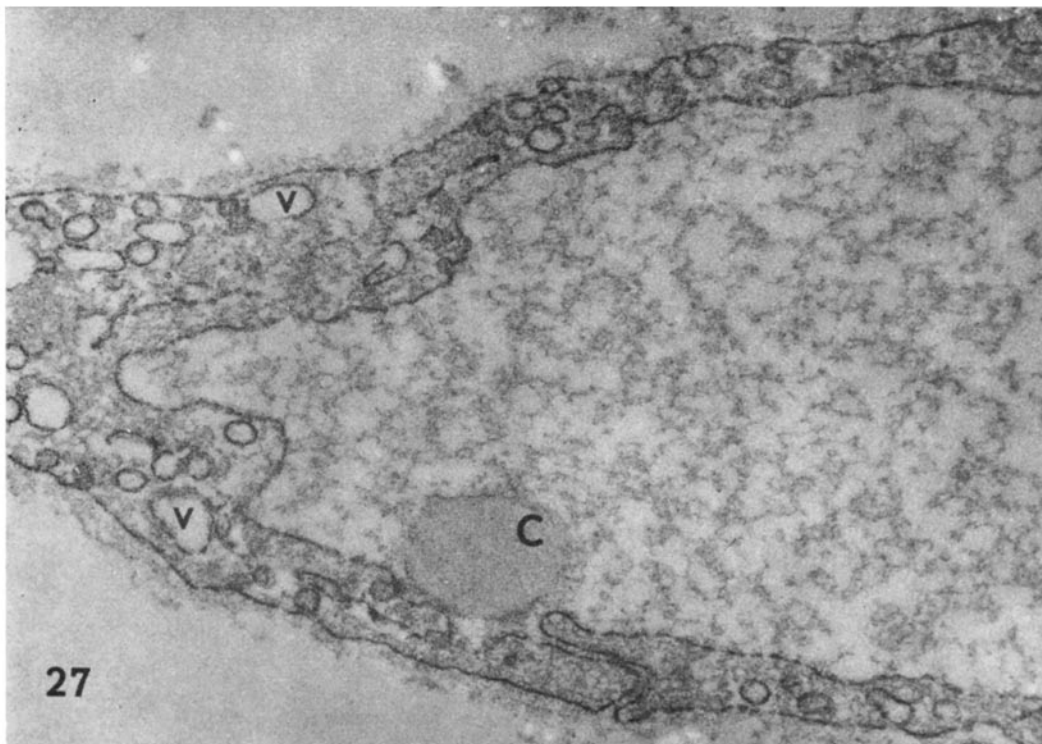


FIGURE 27 Cytochemical reaction in a capillary of a fat pad perfused 10 min with chylomicrons, incubated 1 hr in CaCl_2 -Tris medium in the presence of 0.004 M E-600, and treated 15 min with $\text{Pb}(\text{NO}_3)_2$ -cacodylate solution. An intact chylomicron (C) is present at the luminal surface. Note the intra-endothelial vacuoles (V) and the absence of electron-opaque precipitate. Stained section. $\times 54,000$.

FIGURE 28 Cytochemical reaction in a fat pad prepared as in Fig. 27. Note the small lamellar precipitate (L) outlining an amorphous mass present in the extracellular space, between a fat cell (A) and an elongate connective tissue cell (Ct) near the capillary endothelium (E). Stained section. $\times 61,000$.

refed rats (41). The chylomicrons used contained triglyceride labeled with glycerol-³H. Radioautographic reactions were seen over chylomicrons within the capillary lumen and attached to endothelium, and over fat droplets of fat cells. Reactions unassociated with visible lipid particles were also seen over endothelium and over interstitium near blood vessels. Although these findings suggest that glycerides cross from the blood stream to the fat cells, fat cells probably contain sufficient glycerol kinase (9, 33, 36, 41) to enable them to incorporate into glyceride some of the free glycerol-³H released from chylomicrons. Thus, some, if not all, of the radioautographic reactions seen over the droplets of fat cells could represent newly synthesized glyceride. The radioautographic reactions seen over endothelium and interstitium, however, were probably produced by chylomicron glyceride since glyceride is not released by fat cells (42). These radioautographic findings support the conclusion that glycerides are transported across the capillary endothelium to the subendothelial space.

Triglyceride uptake (1, 25) and lipoprotein lipase activity in mammary tissue (15, 23, 29, 30) are both greatly increased during lactation. Studies in guinea pigs have shown that blood triglyceride taken up by mammary tissue is hydrolyzed to FFA and glycerol (24). Nonsuckling overnight markedly lowers the level of lipoprotein lipase activity in mammary tissue (15, 23) and causes severe hypertriglyceridemia (15). These observations strongly suggest that lipoprotein lipase is also involved in uptake of blood triglyceride by mammary tissue. Morphological aspects of the uptake of chylomicrons and artificial triglyceride particles (Intralipid, Vitrum AB, Stockholm) by lactating mammary tissue have been studied in the rat, mouse, and guinea pig by Schoeffl and French (39). They found that chylomicrons and artificial lipid particles were concentrated along and adhered closely to the luminal surface of the capillary endothelium. Both types of particles were seen also within endothelial vacuoles and multivesicular bodies, but they were not found in pinocytotic vesicles and intercellular junctions, on the outer surface of the endothelium, or in the surrounding connective tissue spaces. Numerous vesicles and strings of vesicles were present in the endothelium, and they appeared sometimes to be related to sites of contact between the lipid and the cell membrane.

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showed that hydrolysis of chylomicrons and artificial triglyceride particles during incubation occurred within the capillary lumina, but not in endothelial cells or in the space beyond the endothelium (39). Many of the lipid particles in the capillary lumina were replaced by large electron-lucent areas that often contained electron-opaque material. These signs of hydrolysis were not seen in tissues incubated in the presence of E-600. The tissues used for these studies were exposed and irrigated *in situ* with cold glutaraldehyde solution for several minutes before they were excised and fixed *in toto*. It is possible, then, that intraluminal hydrolysis of chylomicrons during incubation in these tissues was catalyzed by lipoprotein lipase released during the long interval between stopping the blood flow and fixing the tissue. Whether intraluminal hydrolysis occurs *in vivo* in mammary tissue requires further study.

The morphological and cytochemical findings described above, in conjunction with earlier observations (35, 36, 39, 41), suggest the following mechanism for the transport of fatty acids from chylomicron triglyceride to fat cells of adipose tissue. Chylomicrons in the blood stream are partially enveloped by capillary endothelium and reduced in size by action of lipoprotein lipase. The glycerides are then taken into vacuoles and microvesicles, and hydrolyzed further by the lipase. The contents of the vacuoles, including glycerides and FFA, are released directly, or first transferred to microvesicles and then released, to the subendothelial space. Breakdown of glyceride to FFA continues in microvesicles and is completed in the subendothelial space. Thus fatty acids of chylomicrons cross the capillary endothelium as glycerides and FFA, and cross the extravascular space to fat cells as FFA.

The cytochemical findings show that lipoprotein lipase is present in the capillary endothelial cells of adipose tissue. Although fat cells are thought to be the main source of this enzyme in adipose tissue (6, 27, 32, 35), the manner in which the enzyme could be transferred to the endothelium has not been determined (32). The relatively large amount of hydrolysis observed in the subendothelial space near pericytes suggests that they are also involved in the regulation of lipoprotein lipase activity in adipose tissue. Pericytes could serve either by storing and converting to a "functional" form lipoprotein lipase produced by fat cells (32), or by synthesizing the enzyme *de novo*.

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