Metabolism of cholesterol-enriched chylomicrons. Catabolism of triglyceride by lipoprotein lipase of perfused heart and adipose tissues

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Abstract The chemical and biochemical properties of cholesterol-enriched and cholesterol-poor chylomicrons from rat lymph have been compared. The enriched particles, prepared from cholesterol-containing lipid dispersions, passed into the duodenum, had four to ten times the cholesteryl ester content of the control chylomicrons but had the same content of total "core" (cholesteryl ester + triglyceride) lipid. Both chylomicron species had the same protein composition, the same phospholipid composition, and the same composition of triglyceride fatty acids. The rate of hydrolysis of chylomicron triglyceride for enriched and control particles was determined using both soluble and membrane-supported lipoprotein lipase (LPL) species from heart and adipose tissues. The lipase that was functional in the isolated perfused heart showed no significant difference in initial catabolic rate with cholesterol-enriched and control chylomicrons. The same result was obtained with this isolated LPL species in vitro. The lipase that was functional in isolated perfused epididymal adipose tissue showed a slightly lower catabolic rate with cholesterol-enriched particles (84% of that obtained with control chylomicrons). The same result was obtained with isolated adipose tissue LPL. It is concluded that cholesteryl ester content of chylomicrons under these conditions neither affects their protein composition nor has a major effect on their rate of reaction with lipoprotein lipase.

Supplementary key words remnant lipoproteins

Chylomicrons, secreted into the plasma after triglyceride feeding, are catabolized in the peripheral tissues by lipoprotein lipase (LPL) with the production of remnant lipoproteins (1). As triglyceride is removed from the chylomicrons by lipase activity, their hydrolysis rate with the enzyme decreases. The triglycerides of remnant lipoproteins become at the same time increasingly ineffective as competitors with those of newly-secreted, intact chylomicrons as substrates for the enzyme (2). Remnants differ from intact chylomicrons in both lipid and protein composition. As triglyceride hydrolysis proceeds, remnants become progressively enriched in other lipids, such as cholesteryl ester, relative to their triglyceride content (2–4). However, remnant formation is also accompanied by changes in protein composition, including the content of the lipase coprotein (apoC-2) which is a component of the substrate lipoprotein moiety. When chylomicrons enter the plasma compartment from the lymph they acquire a several-fold excess of coprotein (5). As lipolysis proceeds, the C-apoproteins are lost from the remnant particle (4, 6) and appear in the plasma high density lipoprotein fraction. It has remained unclear whether it is the changing lipid or the protein composition of chylomicron remnant particles that is the primary determinant of their catabolic rate.

An alternative approach to determine the relative importance of lipid and protein composition in LPL reactivity is to follow the initial catabolism of intact particles that have a cholesteryl ester content similar to that of remnant particles. These can be obtained after cholesterol feeding, since dietary cholesterol is carried in the chylomicron particles almost exclusively as cholesteryl ester. In the present research such particles have been used as substrates for LPL in perfused rat heart and adipose tissues.

MATERIALS AND METHODS

Preparation of chylomicrons

Chylomicrons were obtained from male Sprague-Dawley rats (300–350 g) fasted overnight (7). Cholesterol-enriched or control lipid dispersions were delivered by a polythene cannula to the duodenal lumen at a flow rate of 1.5 ml/hr. Fatty acid was supplied either as the unesterified acid or as triglyceride, in each case as a mixture of palmitic and oleic acids (Sigma, St. Louis, MO) in a weight ratio of 0.3 and at an inflow

Abbreviation: LPL, lipoprotein lipase.

concentration of 0.15 mmol/ml. Fatty acid was dispersed with taurocholic acid (0.067 mmol/ml) and triglyceride with lecithin (0.05 mmol/ml). Both infusions also contained bovine serum albumin (0.15 μmol/ml) in 0.15 M NaCl, adjusted to pH 8.0 with 1 M NaOH. Chylomicron triglyceride was labeled when ^{3}H- or ^{14}C-labeled fatty acid was infused. In addition, dispersions for the preparation of cholesterol-enriched chylomicrons contained cholesterol (Pfanstiel, Waukegan, MI) at a concentration of 0.05 mmol/ml. Preliminary experiments showed that chylomicrons collected 3 hr after the initiation of perfusion contained a constant proportion of cholesterol by weight for at least a further 15 hr and this collection period (3–18 hr) was then used in the other experiments.

To purify chylomicrons from the whole lymph, 2 ml of lymph containing 2% (w/v) sucrose was layered under 8 ml of Krebs-Ringer buffer containing 0.005 M disodium-EDTA, pH 7.4, and centrifuged for 9.5×10^4 *g*-av.-min. The floating fat was collected in the upper one ml and discarded, and the major lipid fraction was then recovered in the supernatant after a second centrifugation for 0.61×10^6 *g*-av.-min. Further fractionation was made either by repeating the second ultracentrifugation step or by passing the material through a column of Bio-gel A-150M agarose (Bio-rad, Richmond, CA) equilibrated in the same buffer. The chemical and kinetic properties of chylomicrons prepared by these methods did not differ significantly, as is described under Results.

Perfusion of isolated heart and epididymal adipose tissue

Perfusion of the heart was by retrograde aortic (Langendorff) flow from the dorsal aorta, using the recirculating system (7). Perfusion medium (15 ml) was recirculated at 37°C at a flow rate of 4.0 or 8.0 ml/min. The perfusion medium contained 0.1–1.0 μmol/ml of chylomicron triglyceride, 3% (w/v) of bovine serum albumin (pH 7.4) previously dialyzed overnight against 200 vols of 0.15 M NaCl, 10% of triglyceride-depleted rat plasma ($d > 1.006$ g/ml, from recalcified citrated rat plasma), and inorganic salts to a final medium concentration corresponding to Krebs-Ringer-bicarbonate buffer (0.12 M NaCl, 0.023 M KCL, 0.003 M $CaCl_2$, 0.001 M NaH_2PO_4, 0.001 M $MgSO_4$, and 0.025 M $NaHCO_3$), which was maintained at pH 7.4 ± 0.05 by continuous gassing with 95% O_2–5% CO_2 at 37°C. In experiments to determine the competitive removal of cholesterol-poor and cholesterol-enriched chylomicrons, mixtures of these particles containing alternative radioactive labels in the fatty acid moiety (^{3}H- or ^{14}C-) formed the circulating lipoprotein substrate for heart membrane-supported LPL (2). Sequential samples were taken at 2-min intervals from the reservoir during recirculation. The triglyceride content of 0.2-ml samples was separated from total medium lipids (8) by automated thin-layer chromatography (AIS Multispot, Libertyville, IL) on silica gel layers on glass plates developed in hexane–diethyl ether–acetic acid 83:16:1 (v/v/v). The triglyceride-containing area (visualized with iodine vapor) was removed and radioactivity was determined by liquid scintillation counting (7). The triglyceride removal rates from control and cholesterol-enriched particles were determined from the linear regressions of substrate concentration against time using a Hewlett-Packard 9100 desk-top computer.

Epididymal fat pads were perfused essentially as previously described (9, 10) except that in the present experiments the spermatic artery was perfused directly. A small incision (0.5–1.0 mm) was cut in the aorta and a cannula, consisting of polyethylene tubing (ID 0.011″, OD 0.024″) (IntraMedic PE 10, Clay Adams, Parsippany, NJ) seated to a blunt-ended 1-cm steel tube (30 gauge) (Popper, New Hyde Park, NY), was threaded through into the spermatic artery. Perfusion without recirculation was with a medium identical to that described above for the perfused heart except that it contained insulin (Lilly, Iletin) (100 μU/ml). Triglyceride removal was determined from the substrate concentrations of the reservoir and outflow (9). In the case of both heart and adipose tissues, each preparation was normally used for three separate experiments, each lasting about 15 min, in order to measure the removal of triglyceride from mixtures of different proportions of lipoproteins. Under these conditions, the level of functional enzyme in each tissue at the vascular surface was maintained constant (7, 9).

Lipid and protein analysis

After extraction (8), lipids were fractionated by thin-layer chromatography. Neutral lipids were separated using the system described above. Phospholipids were separated by development in chloroform–methanol–water 65:35:5 (v/v/v), and then extracted from the gel with chloroform–methanol 1:1. Recovery of all lipids was >97%. Lipid analytical methods (11–14) were those previously utilized for the assay of unesterified fatty acids, triglyceride, cholesterol, and phospholipids. Protein was measured by the method of Lowry et al. (15).

The composition of urea-soluble protein of control and cholesterol-enriched chylomicrons was determined by polyacrylamide gel electrophoresis in the presence of 8 M urea (16). Gels (7.5% polyacrylamide) (w/v)

TABLE 1. Composition of cholesterol-enriched and control chylomicrons (weight %)

	Triglyceride	Cholesteryl Ester	Free Cholesterol	Phospholipid	Protein[a]
Cholesterol-enriched	83.9 ± 2.1	6.1 ± 1.9	1.0 ± 0.2	7.7 ± 0.3	1.5 ± 0.3
Control	88.2 ± 1.0	1.1 ± 0.3	0.7 ± 0.2	7.8 ± 0.5	1.4 ± 0.2

[a] Total protein is considered to consist of TMU-soluble apolipoprotein + B-apoprotein (22).

Means ± SD for six preparations. Determination of component lipid classes was as previously described by Fielding et al. (20). Range of cholesteryl ester content was 3.5–8.5% by weight and triglyceride was 81.5–86.2% by weight in the cholesteryl ester-enriched particles.

were developed at 2 mA/tube and stained with Coomassie Blue at 70°C. In some experiments the chylomicrons were preincubated with 10–100% (v/v) rat plasma before reisolation by column chromatography and urea gel electrophoresis (5). Chylomicrons were incubated at a concentration of 3 mg triglyceride/ml with the calculated volume of triglyceride-depleted plasma for 7 min at 37°C.

To determine triglyceride fatty acid composition, 50–150 μg of this fraction, extracted from chylomicrons, was dried under nitrogen, then mixed with 5.0 ml of 1% H_2SO_4 (w/v) in methanol and heated for 60 min at 70°C. After addition of 5 ml of distilled water and 2 ml of heptane, the phases were separated and aliquots of heptane phase were dried down, redissolved in diethyl ether, and the component fatty acid methyl esters were separated by gas–liquid chromatography on 12% ethylene glycol succinate on Chromosorb W. (17).

Particle size determination

Particle size distribution of control and cholesterol-enriched chylomicrons was determined on samples of purified lipoproteins that had been negatively stained with phosphotungstate (18). Grids were photographed after examination in the Siemens 101 electron microscope at 50 kV and photographed at an initial magnification of 22,000. The size of visualized chylomicrons was determined using a Nikon 6C profile projector. Values cited are the means of duplicate analyses of two preparations each of control and enriched particles (200 particles per field).

Isolation and assay of lipoprotein lipase

The high- and low-affinity fractions of LPL from postheparin plasma were isolated by flotation and chromatography (19). These lipase species, with molecular weights of 37,500 and 69,250, respectively, have been shown to correspond to activities released by heparin from heart and adipose tissues (7, 9). Postheparin plasma was obtained 5 min after the intravenous injection of heparin (sodium heparin, Riker, Northridge, CA) (100 IU/kg body weight) into male rats. The plasma (which contained 40–60 enzyme units[1]/ml) was fractionated as previously described (19, 20). Low-affinity lipoprotein lipase (purified about 14,000-fold from plasma total lipolytic activity in a yield of 8–10%) had a final specific activity of about 10,000 lipase units per mg protein, corresponding to a catalytic rate of 200 sec^{-1} with synthetic triglyceride substrate. High-affinity lipoprotein lipase, purified 5000-fold, had a specific activity of 3500–4000 lipase units per mg protein, corresponding to a catalytic rate of 40 sec^{-1} with synthetic triglyceride substrate at pH 8.3. The corresponding values for activity with control chylomicron substrate, pH 7.4 in the absence of heparin were 80 sec^{-1} and 30 sec^{-1} for high and low molecular weight lipases, respectively. Both species were homogeneous by polyacrylamide gel electrophoresis.

Lipolytic activity with solubilized enzyme species was determined in the same balanced-salts medium used for the perfusion of isolated rat heart and adipose tissue preparations. Purified LPL was added at a concentration of 0.05–0.1 lipase units/ml. Assays were made for each chylomicron preparation for at least six substrate concentrations in duplicate. Assay was for 30 min at 37°C. Production of unesterified fatty acid was linear under these conditions and the major kinetic constants (catalytic rate, k_{cat}, and apparent Michaelis constant, $Km_{(app)}$) were determined from the double reciprocal plots ($1/v$ vs $1/S$) by least squares.

RESULTS

Chemical properties of control and cholesterol-enriched chylomicrons

The lipid and protein composition of chylomicrons purified from lymph as described under Methods is shown in **Table 1.** The mean content of cholesteryl ester was increased about seven-fold when cholesterol was included in the medium entering the duodenum.

[1] One enzyme unit catalyzes the release of one μmole fatty acid from triglyceride substrate/hr at 37°C, pH 8.3.

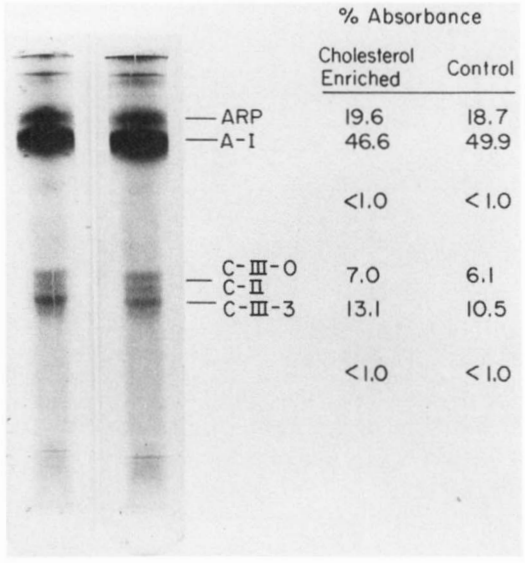

Fig. 1. Protein composition of (left) cholesterol-enriched (7.4% cholesteryl ester by weight) and (right) control (0.9% cholesteryl ester by weight) lymph chylomicrons. Each polyacrylamide gel shows the staining pattern generated from the protein content (extracted with an equal volume of tetramethylurea (22)) from 5.6 mg of total core lipid (triglyceride + cholesteryl ester). Component protein species are identified as previously (ref. 5) and cited values for component staining bands are percent total staining material obtained using an integrating gel scanner (Clifford Instruments, Model 445, Natick, MA).

However the total content of nonpolar lipids (triglyceride + cholesteryl ester) was not significantly different between the two chylomicron preparations (90.0% for cholesterol-enriched particles vs. 89.3% for control particles). The major phospholipid was lecithin (73.5% for enriched and 76.7% for control particles, increased to 80.6% and 80.7% after incubation with 100% plasma). Incubation with plasma also removed the slightly higher content of free cholesterol in the cholesterol-enriched chylomicrons, and after incubation the only substantial difference between the two classes of particles was in their cholesteryl ester content.

Determination of the chylomicron fatty acids showed that in each case the triglycerides contained the infused fatty acid species in approximately the weight ratio delivered to the duodenum (21) (palmitate/oleate ratio to the duodenum, 0.30; for the cholesterol-enriched chylomicrons 0.25, and for the control particles 0.24). Cholesterol-enriched and control particles also showed no significant differences in their content of minor amounts of stearic and linoleic acids.

Particles purified from the lymph of cholesterol-fed animals had a mean diameter of 1822 ± 434 Å and from control animals 1905 ± 418 Å when these were purified by column chromatography on Biogel 150-M (1% crosslinking) as described under Methods. When purification was by centrifugation, the corresponding values were 1861 ± 450 Å for cholesterol-enriched and 1776 ± 486 Å for control chylomicrons. Two different preparations of each substrate prepared by this method gave values of mean diameters differing by less than 5%.

Not only was the total protein of cholesterol-enriched and control chylomicrons the same but there was also no major or consistent difference in the proportions of component urea-soluble apoproteins between the two species (**Fig. 1**). The same result was obtained with chylomicrons that had been preincubated with rat plasma. Absorbance of individual stained bands in these experiments was shown to be linear over at least a 2.5-fold range of protein concentrations.

Triglyceride catabolism in the isolated perfused heart

When mixtures of control and cholesterol-enriched chylomicrons of known composition were perfused through the isolated heart, removal of each species was in proportion to the concentration of these in the perfusion medium (**Fig. 2**), indicating that membrane-supported heart (high-affinity) LPL had the same catalytic rate with cholesterol-enriched and control chylomicron substrates. Similar results were obtained when mixtures containing different proportions of enriched and control particles were passed in succession through the heart (**Table 2**). The experimental removal rates obtained corresponded closely

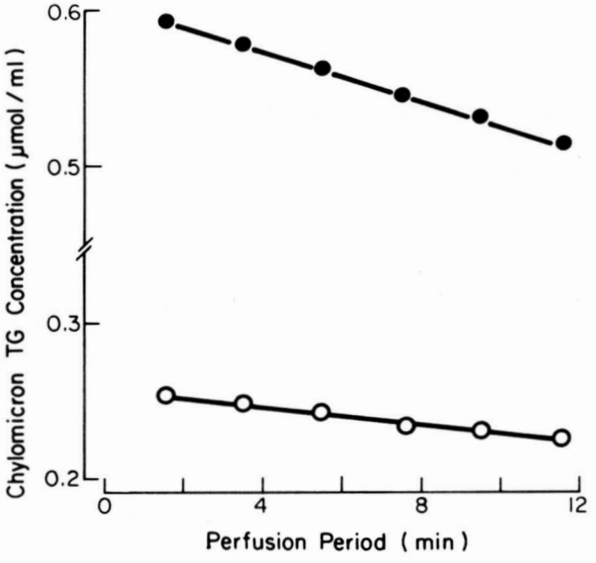

Fig. 2. Triglyceride catabolism in the isolated perfused heart of a mixed substrate of control (● —— ●) and cholesterol-enriched (○ —— ○) chylomicrons. Initial concentrations of substrates were 0.61 and 0.26 μmol/ml respectively (ratio 2.36:1) and removal rates were −0.0082 and −0.0034 μmol/min respectively (ratio 2.41:1).

TABLE 2. Hydrolysis of triglyceride from cholesterol-enriched and control chylomicrons by the isolated perfused heart

Lipoprotein Concentration		Relative Hydrolysis Rate		
Cholesterol-enriched	Control	Calcd (a)	Exptl (b)	Ratio (b/a)
mM triglyceride				
0.038	0.009	4.10	4.04	0.99
0.081	0.040	2.02	2.06	1.02
0.056	0.038	1.49	1.54	1.03
0.022	0.034	0.66	0.70	1.05
0.026	0.048	0.54	0.53	0.98
0.015	0.071	0.22	0.23	1.04
				1.02 ± 0.03

Perfusion was without recirculation using six successive reservoirs in the same perfused heart. Removal rates were calculated from the difference between reservoir and outflow concentrations. Percent triglyceride removed across the circulation varied between 10% and 24% with different reservoir solutions. Both reservoir and outflow media were determined from quintuplicate samples whose triglyceride content was determined after thin-layer chromatography as described under Methods.

to those calculated on the basis of the ratio of triglyceride in each species.

There was no significant difference in the rate of clearance of chylomicrons prepared by infusion of triglyceride–lecithin or fatty acid–taurocholate dispersions. In a typical experiment, initial concentrations of 0.025 and 0.023 μmol/ml triglyceride (ratio 1.07) from chylomicrons prepared with fatty acid or triglyceride, respectively, were perfused together through the heart. Removal of chylomicron triglyceride in the course of a 12-min incubation perfusion was 0.0117 and 0.0112 μmol triglyceride, respectively (removal ratio 1.05).

Catabolism in isolated perfused adipose tissue

Mixtures of known proportions of control and cholesterol-enriched chylomicrons were perfused without recirculation through isolated epididymal adipose tissue. Samples of the eluate were recovered for comparison of triglyceride radioactivities with those of the reservoir (inflow) medium. As shown in **Table 3,** the proportional removal of triglyceride from cholesterol-enriched chylomicrons was lower (0.82 ± 0.05) ($P < 0.005$) than from control chylomicrons (12 experiments). Removal of triglyceride during passage through the tissue in these experiments was in all cases less than 12% of original (reservoir) concentration.

Triglyceride catabolism with soluble LPL species

The catalytic rate of high and low molecular weight LPL species with control and enriched chylomicrons was determined. Previous results have shown these to have the same kinetic properties as LPL released by heparin from perfused adipose and heart tissues respectively (19). Linear double-reciprocal plots were obtained that permitted calculation of both k_{cat} and $Km_{(app)}$ for each of the experimental conditions. While $Km_{(app)}$ values did not differ significantly from those previously reported for either high or low molecular weight LPL, this was not the case for their catalytic rate constants. In the case of high-affinity LPL in solution, rates of activity obtained with control and cholesterol-enriched chylomicrons differed by less than 5%. However, with soluble adipose tissue lipase, the rate of reaction with the cholesterol-enriched particles was significantly lower (**Fig. 3**) (0.79 ± 0.02, three experiments).

TABLE 3. Hydrolysis of triglyceride from cholesterol-enriched and control chylomicrons by isolated perfused adipose tissue

Lipoprotein Concentration		Relative Hydrolysis Rate		
Cholesterol-enriched	Control	Calcd (a)	Exptl (b)	Ratio (b/a)
mM triglyceride				
0.067	0.032	2.07	1.69	0.82
0.050	0.047	1.06	0.91	0.85
0.034	0.073	0.46	0.38	0.82
				0.83 ± .02

Perfusion was without recirculation using three successive reservoirs in the same preparation of isolated epididymal adipose tissue. Removal rates were calculated as described in Table 2. Percent triglyceride removed across the tissue varied between 8 and 12% with different reservoir solutions.

DISCUSSION

There are two potential sources of cholesterol-enriched dietary lipoproteins. These are, first, intact particles secreted cholesterol-rich into the plasma from the lymph after cholesterol feeding; and, sec-

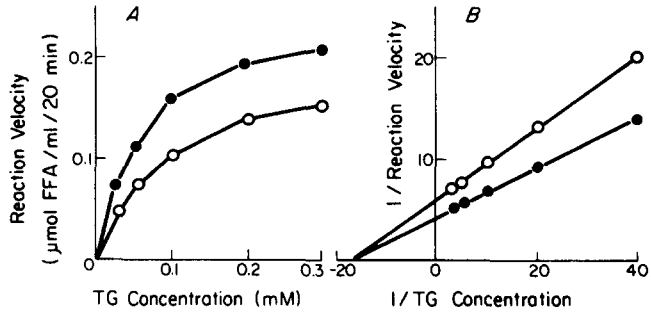

Fig. 3. Kinetics of triglyceride catabolism in vitro from cholesterol-enriched (○ – – – ○) and control (● – – – ●) chylomicrons from adipose tissue by high molecular weight (low-affinity) LPL. Reaction was carried out for 30 min at 37°C in the presence of 10% (v/v) triglyceride-depleted plasma as described under Methods.

ond, remnant particles enriched in cholesterol as a secondary effect of the action of LPL on primary chylomicrons containing lower amounts of cholesterol. Remnant formation is associated with a major decrease in reactivity with LPL (2). It is also associated with a relative enrichment of the triglyceride-depleted particles in arginine-rich and B-apolipoproteins (4). These proteins in turn are the major proteins in lipoproteins rich in cholesteryl ester that appear after long-term cholesterol feeding in several animal species (23, 24).

In the present experiments, chylomicrons from cholesterol-fed donors had an approximately six-fold increase in cholesteryl ester content (associated with a corresponding decrease in triglyceride content) (Table 1) but no other difference in lipid or protein composition, before or after incubation with plasma. Specifically there was no enrichment in arginine-rich apolipoprotein (Fig. 1). Since the liver is the major site for synthesis of this polypeptide (25, 26) and since a major part of this apoprotein in chylomicrons is obtained by equilibration (5, 27), it appears likely that the protein composition of the chylomicrons is determined in large part after the chylomicrons enter the plasma and that an increased content of cholesteryl ester per se is not a major determinant for the transfer of arginine-rich protein to chylomicrons.

In this case, since apart from their cholesteryl ester content the composition of the enriched and control chylomicrons is the same, these can be used to determine the effect of this lipid, in natural particles, on the rate of LPL activity. Remnant particles containing a six-fold increase in cholesteryl ester content have a reactivity with LPL of about 0.40 that of the original particle (1, 2). The catalytic constants for enriched and control chylomicrons were determined with high-affinity (low molecular weight) LPL, both membrane-supported in the heart and in solution after isolation from postheparin plasma. There was no significant difference in reaction rate, nor discrimination of cholesterol-rich and cholesterol-poor particles by the lipase of the heart. With the low-affinity (high molecular weight) species from adipose tissue there was in each case a small but significant inhibition of LPL in reaction with the cholesterol-enriched chylomicrons. However, this was much less marked than would be required to explain the decreased reactivity of remnant particles. Thus cholesteryl ester content does not seem to be a major determinant of the rate of LPL with chylomicrons. A small effect was previously found of incorporation of cholesteryl ester into synthetic dispersed substrates of LPL (28, 29). It now appears more likely that this effect is a biophysical one, perhaps dependent upon the means of substrate dispersion and not significant physiologically, at least in the range of composition of normal dietary and remnant particles. In both these studies, unesterified cholesterol was found to be a major inhibitor of LPL reaction with synthetic substrates.

Cholesterol-rich and control chylomicrons have the same content of free cholesterol, but as triglyceride depletion occurs in remnant formation, the chylomicron free cholesterol is retained and hence the cholesterol/phospholipid ratio of the surface layer also rises. At the same time, because chylomicron lecithin and phosphatidylethanolamine are substrates for LPL, the remnant generated by LPL activity also becomes enriched in its phospholipid moiety with spingomyelin. Finally, while the protein composition of cholesterol-enriched and control chylomicrons was the same, in the course of their catabolism C-apoproteins (including the protein cofactor of LPL activity) are lost and can be recovered in the high density lipoprotein fraction. Therefore the remnant has a much lower C-protein content than the intact, enriched particle, and relatively more arginine-rich apolipoprotein. It appears most likely that one or more of these factors is involved in the decreased reactivity of LPL with remnant particles and that dietary cholesterol, released into the plasma as cholesteryl ester, is not a major factor in the regulation of peripheral triglyceride hydrolysis. On the other hand, since the enriched particles used in this study have a similar cholesteryl ester content to human dietary particles (1), they may provide a better model for other aspects of chylomicron remnant metabolism than do the cholesterol-poor particles produced by feeding a pure triglyceride load.

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