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Research Article

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Metabolism of Cholesterol-Rich Chylomicrons

MECHANISM OF BINDING AND UPTAKE OF CHOLESTERYL ESTERS BY THE VASCULAR BED OF THE PERFUSED RAT HEART

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ABSTRACT The rate of uptake of cholesteryl ester from chylomicrons has been determined with the isolated perfused rat heart and both intact and functionally hepatectomized rats. Uptake was found to be proportional to the cholesteryl ester content of the particles. Transfer of cholesteryl ester to other lipoprotein classes of the plasma was negligible under these conditions, and loss of cholesteryl ester from the medium was associated with quantitative recovery in the vascular bed. The uptake mechanism was nonsaturable and independent of the lipoprotein lipase binding site. Compared with receptor-dependent uptake of low density lipoprotein cholesteryl ester by heart endothelium, the chylomicron pathway appears to provide a major proportion of cholesteryl ester cleared from the plasma. Uptake was initially heparin dependent, and cleared lipid was released by 10 μg/ml of heparin; however, lipid taken up rapidly became heparin resistant and was then hydrolyzed slowly with production of unesterified fatty acid. These results are discussed in the context of the possible role of cholesterol-rich chylomicron remnant lipoproteins in atherogenesis.

INTRODUCTION

Studies on the fate of chylomicron lipids have indicated that the major part of the triglyceride content of these particles is catabolized in the extrahepatic tissues. This is through the activity of lipoprotein lipase (LPL)[1] which is functional at the vascular surface (1). Of the other major components of the chylomicron, some of the phospholipid (particularly lecithin and phosphatidyl ethanolamine) is catabolized by LPL (2, 3) and part is transferred to other lipoproteins (4). Some of the chylomicron protein (particularly the C apoproteins) is transferred to the high density lipoprotein class, and some is retained in the generated chylomicron remnant particle (5). Most of the cholesteryl ester (at least in cholesterol-poor particles) is retained in the remnant during peripheral hydrolysis of triglyceride, and is subsequently cleared by the liver (6, 7).

Normal dietary particles contain significantly higher levels of cholesteryl ester (2.5–6.0%) (8) than lymphatic particles obtained after triglyceride feeding. Chylomicrons containing these levels of cholesteryl ester show rates of peripheral lipolysis comparable to those obtained with cholesterol-poor chylomicrons (9). Such cholesterol-rich chylomicrons appear to be suitable substrates for generation of the cholesteryl ester-rich remnant particles that have been suggested as initiators of atherogenesis (10). The present research was undertaken to determine the fate of the cholesteryl ester moiety of cholesterol-enriched chylomicrons.

METHODS

Preparation of chylomicrons. Animal donors were male Sprague-Dawley rats (300–350 g body wt) fasted overnight to deplete triglyceride in the intestinal lymphatic duct. As previously described in detail (11), a fine polyethylene cannula (Dow-Corning silastic, outside diameter 0.047 inch, Dow Corning Corp., Midland, Mich.) was inserted into the main mesenteric lymph duct and a second into the proximal lumen of the duodenum. The animals were placed in restraining cages and maintained for 1–2 h on physiological saline solution passed into the duodenum at a flow rate of 1 ml/h. After establishment of the lymph flow, the saline infusion was replaced with the same solution containing either a mixture of unesterified fatty acids (palmitic and oleic acids at a molar ratio of 0.3) or the same proportions of triolein and tripalmitin, in the presence of 0.3% sodium taurocholate and 2% wt/vol bovine serum albumin, pH 8.0. The total fatty acid concentration was 150 mM, and cholesterol was present at a concentration of 0–50 mM. This dispersion, prepared in a homogenizer, was passed into the duodenal lumen under the conditions described above. Collection of chylomicrons was begun 4 h later, at which time the specific activity of triglyceride and cholesterol in the lymph of

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[1] *Abbreviations used in this paper*: LPL, lipoprotein lipase; LDL, low density lipoprotein.

animals fed radiolabeled fatty acid and cholesterol had become constant. Lymph was then collected for not more than 18 h into ice-cooled tubes. The chylomicrons recovered (which contained 1–10% cholesteryl ester by weight) were fractionated by ultracentrifugal flotation and column chromatography on Biogel 150-M (Bio-Rad Laboratories, Richmond, Calif.), using a 2 × 50-cm column at a flow rate of 20 ml/h. 2 ml of lymph (containing about 30 mg/ml lipid) was layered under 8 ml of buffered EDTA solution (11) and centrifuged for 10 min at 12,500 rpm in the refrigerated Beckman L2-65 preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), using the 40-rotor. The floating fat layer in 1 ml was removed and replaced with the same volume of buffered saline. The major chylomicron fraction was floated by a second ultracentrifugal run for 30 min at 17,500 rpm. The floating particles were finally fractionated by column chromatography or by a second flotation at 17,500 rpm. The physical and biochemical properties of particles prepared by these methods were not distinguishable, as described elsewhere (9).

Incorporation of radioactive cholesteryl esters. The cholesteryl ester moiety of the chylomicrons was labeled either biologically, using [^{3}H]cholesterol in the duodenal infusate, or synthetically, with a modification of a method described for plasma low density lipoprotein (LDL) (12). Chylomicrons labeled by these methods were catabolized at identical rates, as described below. Commercial [1-^{14}C]-cholesteryl oleate was repurified by thin-layer chromatography on silica gel layers on glass plates developed in cyclohexane-benzene 4/1 vol/vol. The steryl ester band (R_f 0.4) was extracted into benzene with a recovery of 87–95%. 10 μCi of cholesteryl ester (sp act 60–100 μCi/mg) was dissolved in 1.0 ml of dimethyl sulfoxide and injected through a 27-gauge needle into 6 ml of 0.154 M NaCl-0.1% wt/vol sodium-EDTA, pH 7.0, and at 25°C. The optically clear solution was mixed rapidly with 3 ml of a chylomicron solution containing 5–10 mg triglyceride (0.5–1.0 mg cholesteryl ester). After incubation for 60 min at 37°C, the solution was dialyzed at 4°C against two changes (500 vol) of saline-EDTA, then finally layered under saline and concentrated by centrifugation as described above. Recovery of radioactivity in the chylomicron cholesteryl ester fraction was about 20% and subsequent analysis showed that >99.4% of radioactivity was associated with the cholesteryl ester band by thin-layer chromatography. Recovery of chylomicron protein and triglyceride was in each case 50–60% at the flotation step. Recovery of radioactivity increased to about 50% when the incubation period was increased to 3 h, i.e. essentially complete relative to the recovery of triglyceride. With the standard 60-min incubation, the specific activity of cholesteryl ester was unchanged by column chromatography on 1% agarose (in which the particles were included) (9). Specific activity of chylomicrons labeled synthetically was about 10^4 dpm/μg cholesteryl ester. Chylomicrons labeled biologically with 1 mCi of [^{3}H]cholesterol per animal recipient contained by comparison about 10^3 dpm/μg cholesteryl ester.

Preparation of protein-labeled chylomicrons. Protein was labeled biologically by infusion of [^{3}H]leucine (10–50 μCi/ml in the lipid dispersion) into the duodenum. More than 99.8% of radioactivity in the recovered chylomicrons was precipitated by 12.5% trichloroacetic acid. To determine the chylomicron content and apoprotein B radioactivity, purified particles (or perfusate samples purified by chromatography on Sepharose 2B; Pharmacia Fine Chemicals, Piscataway, N. J.) (1) were delipidated with ethanol-diethyl ether (2/1 vol/vol) and diethyl ether at −20°C. Recovery of radioactivity was >97% through the delipidation step. The protein (dissolved in 0.2 ml of 0.01 M sodium phosphate buffer containing 1% wt/vol sodium dodecyl sulfate and 1% 2-mercaptoethanol) was subjected to electrophoresis in 10% polyacrylamide gel with 0.1% sodium dodecyl sulfate in the gel and running buffers (13). The gels were stained in Coomassie Blue (0.02% wt/vol) in methanol/water/acetic acid, 45/45/10 vol/vol, and destained methanol/water/acetic acid, 5/90/5. The visualized B protein band (comigrating with apo B from low density lipoprotein) was removed by slicing, mixed with 0.4 ml of 30% hydrogen peroxide, and kept at room temperature for 24 h to dissolve the gel. The solution was mixed with 10 ml of Aquasol liquid scintillation fluid (New England Nuclear, Boston, Mass.), and radioactivity determined as described below. Recovery in the whole gel of radioactivity through the electrophoresis procedure was >94%.

Perfusion of the isolated rat heart. Retrograde aortic (Langendorff) perfusion was carried out as described (11). Briefly, hearts from male rats fasted overnight were first perfused without recirculation with buffered Krebs-bicarbonate[2] gassed with 95% O_2–5% CO_2, at a flow rate of 4–8 ml/min for 5 min. The organs were then perfused, with or without recirculation, with medium containing albumin or triglyceride-depleted recalcified citrated rat plasma ($d > 1.006$ g/cm^3 infranatant solution; 6% wt/vol protein), glucose (1 mg/ml), and chylomicrons as specified in balanced Krebs-bicarbonate buffer. The rate of clearance of triglyceride or cholesteryl ester in nonrecirculation perfusion was determined from samples taken from the inflow and outflow (perfusate) solutions. Preliminary experiments established that equilibrium conditions of substrate concentration were established within 2 min when the flow rate was 8 ml/min and within 3 min when the flow rate was 4 ml/min. To determine the kinetic characteristics of the removal systems, sequential reservoir solutions containing different chylomicron concentrations were passed through the heart. The apparent Michaelis concentration and maximal velocity of clearance were determined from the slope and intercept of the plot of Ps_i vs. ln $(1\text{-}P)$ where s_i is the inflow substrate concentration and P the proportional removal of substrate across the vascular bed (14). The slope of the plot is $K_{m_{app}}$; for a nonsaturable system the plot is a vertical line because P is constant.

Preparation of remnant lipoproteins. Remnants were prepared using the perfused heart (1). Chylomicrons (initial triglyceride concentration 0.5–1.5 μmol/ml) were recirculated in the presence of plasma proteins, and samples of the remnants generated by endothelial LPL were taken at intervals from the perfusion medium for analysis. Remnants were recovered in the void volume after chromatography on columns (2 × 50 cm) of Sepharose 2B equilibrated with 0.154 M NaCl (1). Recovery of chylomicron triglyceride and cholesteryl ester in the void volume was complete (98–103%).

Preparation of rat lipoproteins. Rat plasma LDL was prepared from the $d > 1.006$ g/cm^3 infranatant solution by ultracentrifugal flotation between the density limits 1.02–1.04 g/cm^3 and high density lipoprotein between 1.07–1.21 g/cm^3. Lipoprotein-depleted plasma was prepared by centrifugation at 1.21 g/cm^3, and the purified lipoproteins and infranatant solution were dialyzed against 0.15 M NaCl–0.01% disodium-EDTA, pH 7.4, before use.

Preparation of functionally hepatectomized rats. Exclusion of the hepatic circulation in animals fasted overnight was achieved with ligatures placed around the anterior and posterior mesenteric and celiac arteries and the hepatic portal

[2] Krebs-bicarbonate solution: 0.118 M NaCl, 0.025 M $NaHCO_3$, 0.0035 M $CaCl_2$, 0.005 M KCl, 0.001 M KH_2PO_4, 0.001 M $MgSO_4$, pH 7.4.

vein. At the same time the carotid artery was cannulated with a silastic (0.048 inch outside diameter) tube (15). The animals were allowed to recover in restraining cages, and a bolus of chylomicron lipid (0.1 μmol triglyceride/ml plasma volume; 4.5% of body wt) was then injected through a femoral vein. At intervals up to 30 min, samples of whole blood (0.25 ml) were withdrawn from the carotid cannula for analysis of lipid radioactivity. Sham-hepatectomized animals were provided only with the carotid cannula. Correction for dilution caused by saline in the carotid cannula was made by determination of the plasma protein content of these samples.

Analytical techniques. Lipoprotein lipids were extracted with a chloroform-methanol mixture (1/2 vol/vol) (16) and after separation of the phases, samples of the chloroform layer were assayed by thin-layer chromatography on silica gel. Plates were developed in hexane-diethyl ether-acetic acid (83/16/1 vol/vol), and the triglyceride (R_f 0.45–0.55) and cholesteryl ester (R_f 0.9–0.95) areas were taken for chemical and radiochemical analysis. In some experiments the areas corresponding to free cholesterol, fatty acid, and phospholipid were also recovered. Extraction of gel areas with 1/1 chloroform-methanol gave complete recovery of each lipid class (11). Free and ester cholesterol, phospholipid (as lipid phosphorus), and triglyceride were determined by standard microprocedures (1). ^3H and ^{14}C were measured with liquid scintillation spectrometry in toluene containing 0.5% PPO, 0.03% POPOP. Quenching was determined with [^3H]- and [^{14}C]toluene. Total protein was determined by a modification (17) of the Lowry procedure (18). Protein radioactivity was determined with Aquasol aqueous scintillation medium.

Materials. [9,10-^3H]- and [1-^{14}C]palmitic acids were purchased from New England Nuclear and the corresponding unlabeled fatty acids from Sigma Chemical Co. (St. Louis, Mo.). [2-^3H]glyceryl trioleate and cholesteryl [1-^{14}C]oleate were purchased from Amersham/Searle, Arlington Heights, Ill. Unlabeled triolein and tripalmitin were from Sigma Chemical. [1,2-^3H]Cholesterol and L-[4,5-^3H]leucine were from New England Nuclear, and albumin was bovine fraction V (Reheis Chemical Co., Chicago, Ill.). Sepharose 2B was purchased from Pharmacia Fine Chemicals, and Agarose 150-M from Bio-Rad. Heparin (intestinal) was purchased from Riker Laboratories, Inc., Northridge, Calif. and Deacidite FF anion exchange resin (Cl-form) was the gift of the Permutit Co., England.

RESULTS

Uptake of chylomicron cholesteryl ester by the perfused heart. When hearts were perfused by recirculation with cholesterol-enriched chylomicrons labeled with [^3H]cholesterol and ^{14}C-fatty acid, there was clearance of both triglyceride (^{14}C) and cholesteryl ester (^3H) radioactivity from these lipid classes after separation by thin-layer chromatography. For particles containing 6% wt/wt cholesteryl ester in a medium of 10% plasma protein vol/vol (0.6% wt/vol plus 5.4% wt/vol albumin), the rate of loss of cholesteryl ester was about 0.5 (0.50±0.06, three experiments) on a percentage basis (0.036 wt/wt) that observed for triglyceride (Fig. 1). These experiments were repeated with chylomicrons labeled biologically with [^3H]-cholesterol, and synthetically with cholesteryl [1-^{14}C]-oleate as described in Methods (Fig. 2). There was no significant difference (four observations) in the removal rates of the two labels, indicating that incorporation of labeled cholesteryl ester with dimethyl sulfoxide provided an appropriate tracer for chylomicron steryl ester metabolism. At the end of the perfusion period, the heart was washed for 5 min with Krebs-bicarbonate buffer to remove perfusion medium, and the level of radioactive lipid retained in the heart was then determined by liquid scintillation counting of the extracted total heart lipid. Recovery of cholesteryl ester radioactivity cleared from the perfusion medium to the heart was 96.5–101.5% (four experiments).

The loss of chylomicron cholesteryl ester was also confirmed by chemical analysis. Intact chylomicrons containing initial cholesteryl ester to triglyceride ratios of 0.05–0.10 (corresponding to an initial content of 4.6–9.4% cholesteryl ester as cholesteryl oleate) were perfused through the isolated heart with

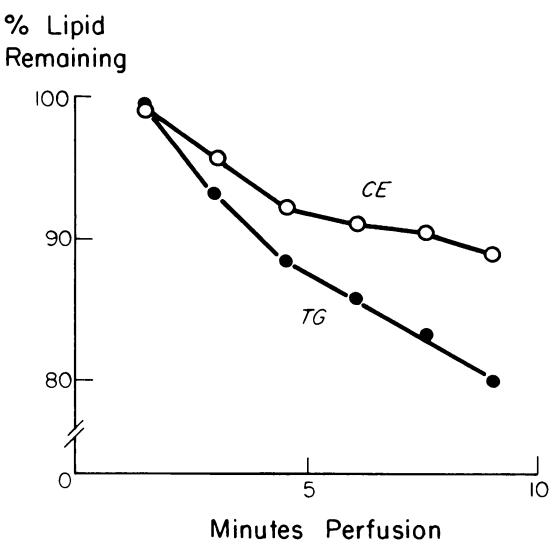

FIGURE 1 Clearance of triglyceride (TG) and cholesteryl ester (CE) from cholesterol-rich (6% wt/wt) chylomicrons in the isolated perfused rat heart. The chylomicrons were labeled with [^{14}C]palmitic acid and with [^3H]cholesterol. Removal of triglyceride and cholesteryl ester was determined as a function of perfusion period in terms of removal of radioactivity in these lipids after isolation by thin-layer chromatography. Using the double-label settings of the scintillation counter overlap of ^{14}C-radioactivity into the ^3H-channel was about 8.5% and overlap of ^3H into the ^{14}C-channel was about 0.5%. Channel overlap and quench corrections were made automatically with a programmed Hewlett-Packard 9100 B desktop computer (Hewlett-Packard Co., Palo Alto, Calif.). Perfusion was carried out in a recirculating volume of 15 ml, in the presence of 10% vol/vol plasma proteins (0.6% wt/vol + 5.4% wt/vol albumin), and with an initial substrate concentration of 88 μg/ml triglyceride, 5 μg/ml cholesteryl ester. Values represent the means of duplicate determinations, expressed in terms of concentrations at 2 min after the initiation of perfusion. In three experiments under the same conditions, the ratio of cholesteryl ester to triglyceride removed was 0.50±0.06.

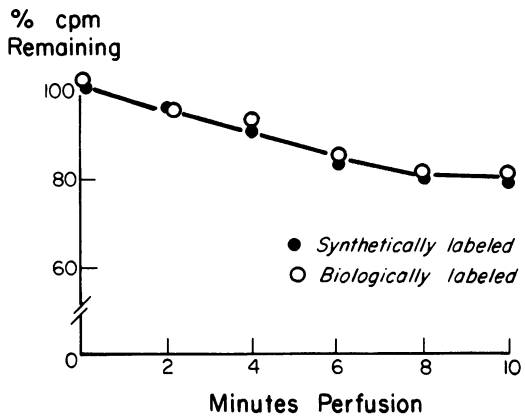

FIGURE 2 Comparison of the rates of clearance of biologically and synthetically labeled cholesteryl ester from chylomicrons in the perfused rat heart. Chylomicrons were labeled with [^{3}H]cholesterol by luminal infusion of the chylomicron donor, then by incubation with cholesteryl [1-^{14}C]oleate as described in Methods. The perfusion conditions were as described for Fig. 1. Values represent the means of duplicate determinations at the intervals stated. Removal rates in four experiments were 0.99±0.02 (synthetically/biologically labeled).

recirculation at an initial triglyceride concentration of 1.0 mM. Samples were taken for analysis as shown in Table I. Remnant formation was associated with loss of triglyceride, phospholipid, and protein as previously described (1) but also in this case significant amounts of cholesteryl ester. From the cholesteryl ester/triglyceride weight ratios of the remnants isolated by chromatography, it can be calculated that approximately half the sterol ester content of the chylomicrons was lost from the particles in the course of loss of 80% of triglyceride content and a similar proportion from smaller (95%-depleted) remnants. Incubation of chylomicrons with perfusion medium over the same time period resulted in no detectable loss of cholesteryl ester when the particles were subsequently isolated by Sepharose gel chromatography and chemically analyzed, and <1% of chylomicron cholesteryl ester radioactivity (0–0.9%) (three experiments) was included in the column fractionation volume under these conditions, indicating negligible transfer of steryl ester to other lipoprotein fractions under these conditions.

Effect of heart LPL on chylomicron cholesteryl ester. LPL was released from the isolated perfused heart by heparin (10 μg/ml) in a perfusion medium containing 10% plasma proteins. Heparin was then removed from the eluate (as this is not a component of the serum protein) by passing the perfusate through a column (0.9 × 10 cm) of Deacidite ion-exchange resin (11) equilibrated with 0.1 M sodium phosphate pH 7.0. Recovery was 50–60%. The eluate was dialyzed against 500 vol 0.154 M NaCl, then was incubated with chylomicrons labeled in both triglyceride and cholesteryl ester moieties. As shown in Fig. 3, hydrolysis of chylomicron triglyceride was not accompanied by any hydrolysis of chylomicron cholesteryl ester.

Effect of perfusion on chylomicron protein. Chylomicrons labeled in the triglyceride and protein moieties were perfused with recirculation in the isolated heart. As shown in Fig. 4, in the course of lipolysis of chylomicron triglyceride there was no loss of radioactivity from the protein moiety. In samples of perfusion medium treated with an equal volume of 25% wt/vol trichloroacetic acid, there was no loss of radioactivity from chylomicron protein detectable as acid-soluble material.

When delipidated chylomicron proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate, the apo B protein band (which cochromatographed with immunoreactive B protein from chylomicrons or rat plasma LDL) contained 32% of total recovered radioactivity (range 30–35%). As shown in Fig. 5, when determination of apo B protein radioactivity was carried out in the course of perfusion of chylomicrons through the heart, loss of triglyceride

TABLE I
Composition of Remnant Particles from Cholesterol-Enriched Chylomicrons

Residual TG content	Composition (% by weight)					CE/TG ratio	
	TG	CE	Protein	FC*	PL‡	Calculated§	Found
%							
100.0	87.2	5.8	2.3	0.8	4.0	—	0.067
57.7	82.2	6.8	3.9	1.0	6.1	0.115	0.083
20.5	74.3	8.5	8.9	1.5	7.0	0.324	0.114
5.8	62.4	13.8	13.0	2.5	8.4	1.142	0.220

Values are the means of duplicate analyses of remnants prepared in the perfused heart in the presence of 10% plasma, after reisolation by chromatography on Sepharose 2B (1). Triglyceride (TG) content was determined as triolein, cholesteryl ester (CE) as cholesteryl oleate, and phospholipid as lecithin (mol wt 750). The residual triglyceride content was determined from the level of circulating radioactive triglyceride (11). In the absence of the heart, incubation of the medium at the same time-points (zero time, 30, 45, and 60 min) gave CE/TG ratios of 0.067, 0.064, 0.066, and 0.067, respectively.

* FC, free cholesterol.

‡ PL, phospholipid.

§ Calculated CE/TG ratio was determined on the basis of zero removal of cholesteryl ester from the chylomicron in the course of triglyceride hydrolysis. In three other experiments, remnants containing 20.0–26.8% residual triglyceride content showed experimental CE/TG ratios of 0.13±0.02 compared with the expected 0.32 for total retention of cholesteryl ester.

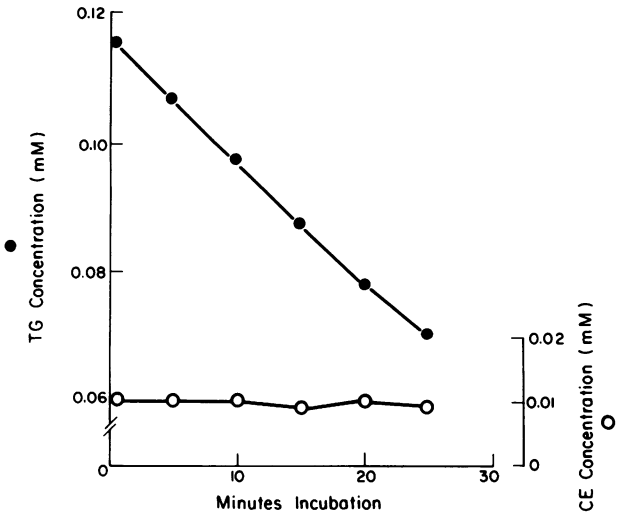

FIGURE 3 Catabolism of chylomicron lipids in vitro after incubation with lipoprotein lipase obtained by perfusion of the isolated heart with heparin (10 μg/ml) then deheparinized by ion-exchange through Deacidite FF in 0.1 M phosphate (11) with an overall recovery of 50–60% of enzyme activity. The chylomicrons were labeled biologically in the triglyceride and cholesterol moieties as described in Fig. 1. Incubation was at 37°C in the presence of Krebs-bicarbonate salt solution, 0.6% wt/vol rat plasma proteins and 5.4% albumin, pH 7.4. Values are the means of duplicate analyses of lipids separated by thin-layer chromatography. No removal of cholesteryl ester was found whether or not heparin was removed. TG, triglyceride; CE, cholesteryl ester.

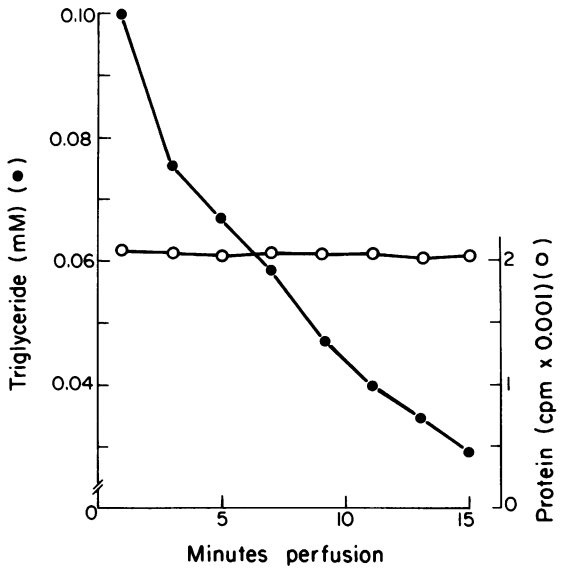

FIGURE 4 Catabolism of chylomicrons labeled in the triglyceride and protein moieties. Cholesterol-rich chylomicrons (6.4% wt/wt cholesteryl ester) were perfused through the isolated rat heart in the presence of 10% vol/vol plasma (0.6% wt/vol plasma protein) and albumin. Duplicate 0.1-ml samples were taken directly into counting medium (Aquasol) for determination of ^{3}H-(protein) radioactivity. Duplicate determinations of triglyceride were made after thin-layer chromatography of extracted lipids as described in Methods. Protein radioactivity in five experiments was 98.3 ±2.7% of initial activity under conditions where mean triglyceride removal was 59.8% and mean cholesteryl ester removal 33.0%.

and cholesteryl ester was not associated with detectable loss of apo B protein radioactivity. No protein radioactivity could be recovered from the heart tissue, indicating that if there were exchange with membrane-associated apoproteins, this was slow compared to the circulation time of the chylomicrons.

Kinetics of cholesteryl ester clearance. Hearts were perfused with chylomicrons labeled in the cholesteryl ester moiety with cholesteryl [1-^{14}C]oleate. The rate of uptake was determined during an initial 10-min perfusion period (Fig. 6). After washing with Krebs-bicarbonate buffer for 10 min, the heart was perfused with a 40-fold excess of unlabeled chylomicrons of the same cholesteryl ester content, and then finally with the labeled chylomicrons at the original concentration together with the same excess of unlabeled chylomicrons. As shown in Fig. 6, there was only a slight (≅10%) decrease in the rate of uptake of cholesteryl ester radioactivity by the heart, indicating that the uptake process was in large part nonsaturable. A further study was made of the simultaneous uptake of cholesteryl ester and triglyceride by the perfused heart. The latter process, mediated by endothelial LPL, was previously shown to be a saturable process with expected Michaelis-Menten kinetics for an apparent K_m of 0.05–0.1 mM triglyceride (11, 14). As shown in

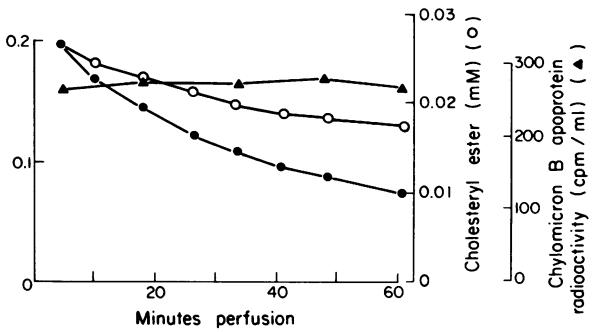

FIGURE 5 The retention of chylomicron apo B protein in chylomicron triglyceride and cholesteryl ester catabolism. Cholesterol-rich chylomicrons (4.6% wt/wt cholesteryl ester) labeled with [^{3}H]leucine and [^{14}C]palmitic acid were perfused through the heart as described for Fig. 4. 2.5-ml samples were taken for protein analysis at the times indicated, the chylomicrons recovered as described in Methods, 0.5 ml of carrier (unlabeled) chylomicrons (10 mg triglyceride/ml) was added, and the mixture delipidated for electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Radioactivity of the apo B protein bands was determined as described in Methods. Radioactivity of triglyceride and cholesteryl ester was determined after lipid extraction and thin-layer chromatography.

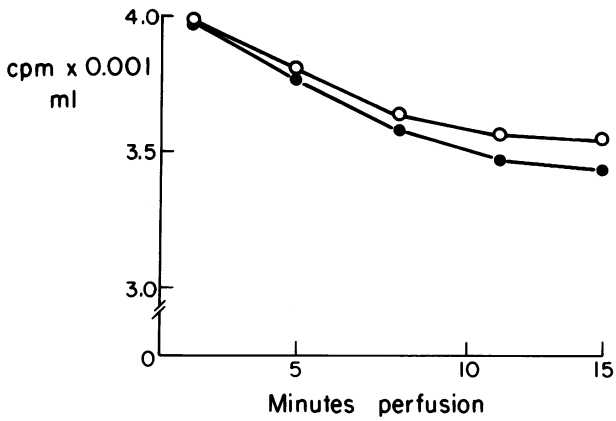

FIGURE 6 Noncompetitive removal of labeled and unlabeled chylomicron cholesteryl ester. The isolated heart was first perfused with cholesterol-rich chylomicrons (8.3% cholesteryl ester wt/wt) at an initial concentration of 3.6 μg/ml cholesteryl ester, labeled synthetically with cholesteryl [1-^{14}C]-oleate (open circles), then after washing with the chylomicrons from the same preparation (unlabeled) for 5 min (147 μg/ml cholesteryl ester), then with a mixture of both labeled and unlabeled chylomicrons at the same concentrations. Loss of cholesteryl ester radioactivity under the latter conditions (40-fold excess of unlabeled chylomicrons; closed circles) was decreased 39–48% (four experiments).

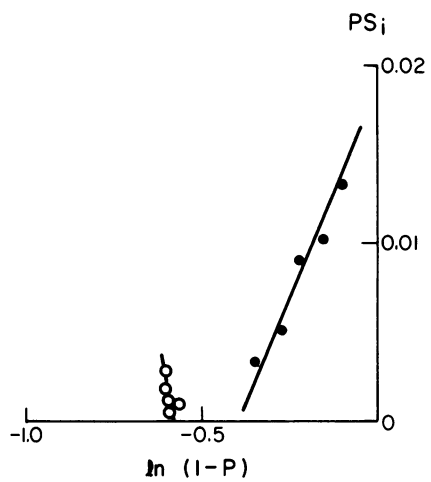

FIGURE 7 Kinetics of removal of triglyceride (●) and cholesteryl ester (○) from cholesteryl ester-rich chylomicrons as a function of medium chylomicron concentration. Reservoirs (passed without recirculation across the vascular bed of the heart at 4.0 ml/min) contained successively 0.01, 0.02, 0.04, 0.07, and 0.10 mM chylomicron triglyceride (0.6, 1.2, 2.4, 4.2, and 6.0 μg/ml chylomicron cholesteryl ester). The particles had been labeled biologically with [^{3}H]palmitic acid and were then labeled synthetically with [1-^{14}C]cholesteryl oleate. Removal across the vascular bed was determined from quadruplicate samples of inflow (reservoir) and outflow (perfusate) media from each medium after an equilibration period of 3.5 min. Proportional removal (P) is where s_i, s_0 are the inflow and outflow substrate ($s_i - s_0/s_i$) concentrations of each lipid fraction. The correlation coefficient at the point of triglyceride removal was $r = 0.96$ and for cholesteryl ester removal 0.84.

Fig. 7, triglyceride hydrolysis from cholesterol-enriched chylomicrons was saturable with an apparent K_m similar to that previously recorded. On the other hand, cholesteryl ester clearance was nonsaturable over this concentration range.

Effect of plasma protein on cholesteryl ester uptake. The level of plasma protein in the perfusion medium was varied from 0 to 6% wt/vol, whereas the total medium protein was maintained at 6% wt/vol by addition of albumin. As shown in Table II, plasma significantly decreased the rate of uptake of cholesteryl ester from chylomicrons, while slightly increasing the rate of triglyceride catabolism by LPL. Uptake in 100% vol/vol plasma was approximately 50% of that observed in the absence of added plasma protein.

When perfusion was carried out with isolated plasma lipoproteins in the presence of lipoprotein-depleted serum, addition of high density lipoprotein significantly inhibited uptake of cholesteryl ester, so that at 200 μg protein/ml (similar to the reported concentration in rat plasma) (19) uptake was only 53±6% (three determinations) that found in the presence of lipoprotein-depleted serum. LDL gave no detectable inhibition at 40 μg/ml, its concentration in plasma (19). Thus, the inhibitory action of plasma appears to be associated in major part with the plasma high density lipoprotein fraction.

Effect of chylomicron cholesteryl ester content. As shown in Fig. 8, the rate of cholesteryl ester uptake from control (cholesterol-poor) or cholesterol-enriched particles was directly dependent upon their cholesteryl ester content at least up to 10% wt/wt cholesteryl ester.

Effect of heparin on cholesteryl ester uptake. Heparin at low concentrations (10 μg/ml) removes the ability of the heart to catabolize chylomicron tri-

TABLE II
Effect of Plasma Proteins on Cholesteryl Ester Clearance

Plasma protein plus Albumin	CE removal	TG removal
mg/ml	*μg/15 min*	*μg/15 min*
0 60	4.2±0.5	111±7.5
12 48	3.8±0.2	130±10.4
36 24	2.6±0.3	138±11.8
60 0	2.2±0.2	160±8.5

Initial triglyceride (TG) concentration in each case was 212 μg/ml and cholesteryl ester (CE) content was 12.2 μg/ml. The perfusion was 15 min, with a flow rate of 8 ml/min. Clearance values represent the difference between the initial and final medium lipid contents assayed in triplicate. Plasma protein represents total protein recovered in the $d > 1.006$ g/cm^3 infranatant solution after centrifugation for 24 h at 40,000 rpm as described in Methods. Values are means±SD of three determinations.

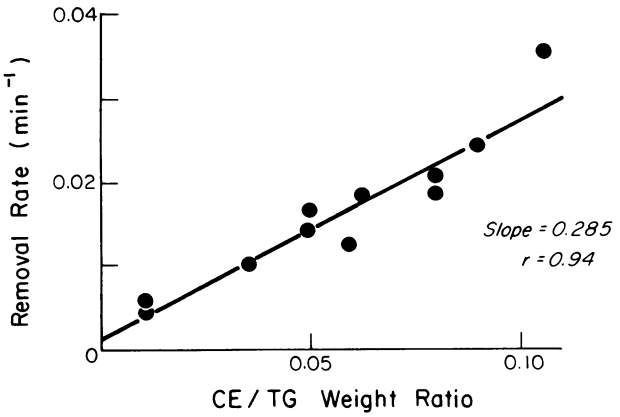

FIGURE 8 The relationship between chylomicron cholesteryl ester content and the first-order removal rate by the isolated perfused heart. Particles were labeled biologically with [^{3}H]cholesterol, and removal rates were determined in the isolated perfused heart in the presence of 0.6% plasma proteins + 5.4% albumin, as described in Methods. CE, cholesteryl ester; TG, triglyceride.

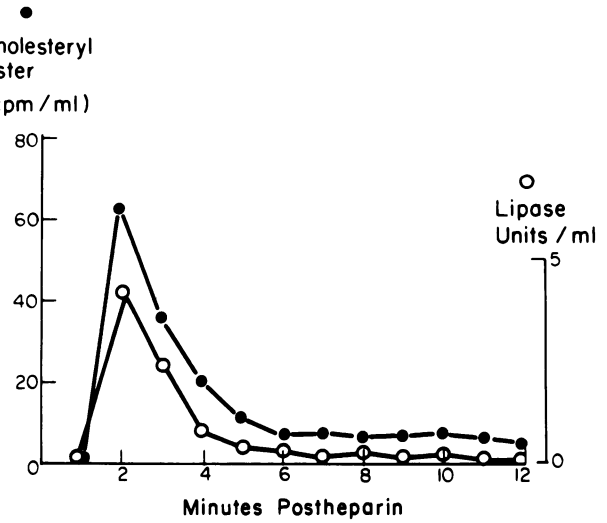

FIGURE 9 The elution of cholesteryl ester radioactivity and lipoprotein lipase from the perfused heart after the passage of heparin (10 μg/ml) in perfusion medium containing 0.6% wt/vol plasma proteins and albumin. The flow rate was 4.0 ml/h. Lipase activity (1 U of activity represents the release of 1 μmol unesterified fatty acid/h) was determined with unlabeled chylomicron substrate at pH 7.4, 37°C, and was assayed titrimetrically (17). Cholesteryl ester radioactivity was assayed in samples of postheparin perfusate after separation of labeled cholesteryl esters by thin-layer chromatography.

glyceride by releasing the functional fraction of lipoprotein lipase from its membrane binding site into the medium (11). As shown in Table III, when heparin was passed through the heart under these conditions there was a loss of about 95% both of ability to catabolize triglyceride and also to clear cholesteryl ester. Additionally, heparin released cholesteryl ester radioactivity into the medium (Fig. 9). The time-course of release of LPL activity and cholesteryl ester radioactivity were similar, possibly indicating release of an enzyme-chylomicron complex from the endothelial surface. As shown in Table IV, almost the whole of heparin-resistant radioactivity from chylomicrons labeled with cholesteryl [1-^{14}C]oleate was recovered in the steryl ester fraction of heart lipids. In a further series of experiments, the heart was perfused for 2 min with chylomicrons containing labeled cholesteryl oleate, then the proportion of cleared radioactivity which was releasable by heparin was determined after nonrecirculation perfusion with Krebs-bicarbonate for periods of up to 30 min. Inasmuch as the cholesteryl ester radioactivity cleared was accurately represented by activity recoverable in the tissue, the proportion of heparin-releasable, cleared cholesteryl ester could be determined by perfusing the heart with heparin-containing medium, then determining the nonreleasable fraction by subsequent direct analysis of total heart lipids. As shown in Fig. 10, the cholesteryl ester cleared by the heart rapidly became heparin resistant so that about 80% of cleared cholesteryl ester was heparin resistant after a 5-min wash period. Although

TABLE III
Effect of Heparin on Triglyceride and Cholesteryl Ester Clearance by the Heart

	Substrate concentration (a)			Removal across tissue (b)			Proportional ratio (b/a)		
	TG	CE	CE/TG	TG	CE	CE/TG	TG	CE	CE/TG
	μg			μg					
Preheparin	135	5.3	0.052	30.8±1.5	0.52±0.06	0.017	0.23	0.10	0.33
Postheparin	130	5.3	0.052	3.9±1.1	0.06±0.03	0.015	0.03	0.01	0.29

Values are means±SD for the removal rates of triglyceride (TG) and cholesteryl ester (CE) across the vascular bed of the perfused heart for a flow rate of 4 ml/min. Values given are the difference between inflow and outflow (perfusate) lipid concentrations determined from quadruplicate assays in each case.

TABLE IV

Balance of Lipid Radioactivity Cleared by the Perfused Heart

Lipid fraction	Cleared from medium	Released by heparin	Retained by tissue
		dpm	
Cholesteryl ester	100,000±2,838	6,262±618	92,764±6,396
Triglyceride	—	32±10	291±58
Fatty acid	—	15±12	2,206±138
Phospholipid	—	12±11	149±118
Total		6,321±620	95,410±6,201

Values (means±SD for four experiments) were obtained after recirculation perfusion for 10 min (perfusion volume 15 ml) in the presence of 5 μg/ml chylomicron cholesteryl ester labeled with cholesteryl [1-^{14}C]oleate, followed by perfusion with heparin (10 μg/ml) for 5 min. Radioactivity of component lipid classes was determined after extraction with chloroform-methanol and thin-layer chromatography as described in Methods. Recovery of cleared radioactivity into the heart was complete (98–103%). When similar experiments were carried out with chylomicrons labeled with [^{3}H]cholesterol, loss of radioactivity from free cholesterol to the heart was about 15% (range 14–16%) that from chylomicron cholesteryl ester of the same cholesterol-specific radioactivity.

most of the cleared radioactivity was recovered in the cholesteryl ester fraction of the heart lipids after perfusion, there was a measurable low rate of hydrolysis, particularly shown as recovery of radioactivity in the unesterified fatty acid fraction, so that after 30 min of perfusion, approximately 2.5% of cleared cholesteryl ester had been hydrolyzed, representing about 75 ng cholesteryl ester. A small amount of radioactivity was found in other lipid ester fractions.

Cholesteryl ester uptake in intact and hepatectomized rats. As shown in Fig. 11, cholesteryl ester in cholesterol-rich chylomicrons was cleared from the plasma of functionally hepatectomized rats with similar kinetics to those found for the isolated perfused heart. The logarithmic rates were 0.012–0.018/min (three experiments), which were maintained over removal of at least 2 h of recirculation. This can be compared with a rate of 0.003/min for the isolated heart in the presence of plasma protein (6% wt/vol) (Table II). Under the same conditions in the intact animal, the rate of removal was 0.09–0.11/min (three experiments), reflecting the major role of the liver in the clearance of chylomicron cholesteryl ester (6, 7).

DISCUSSION

Recent research has identified several pathways by which lipoprotein cholesteryl ester enters cells. Most studies have utilized cells in culture. Attention has been particularly directed toward specific receptors for LDL (20) and for lipoproteins containing arginine-rich apolipoprotein (21). Both receptors involve endocytosis of the lipoprotein particle. The lipoproteins involved in these reactions are relatively small, with diameters of about 200 Å, compared to chylomicrons, whose mean diameter in the present study was 1,900 Å (9). There appears to be no evidence that chylomicrons are taken up by endocytosis or pinocytosis by the vascular endothelium. Rather, the triglyceride moiety is in large part hydrolyzed at the vascular surface by LPL, and the remnant particle is cleared by the liver (6, 7). In previous studies on remnant formation, which utilized cholesterol-poor particles (1), most cholesteryl ester was retained in the remnant. The present study now indicates that in cholesterol-rich chylomicrons there is major uptake of cholesteryl ester from the chylomicron into the cells of the coronary bed at a rate proportional to particle cholesteryl ester concentration. The low rates of clearance observed

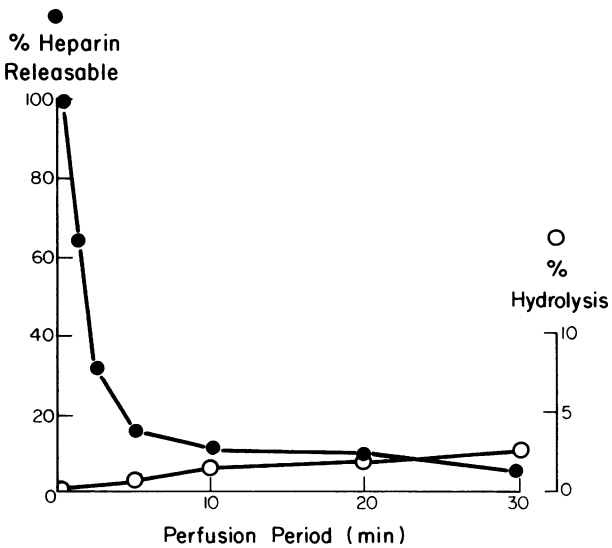

FIGURE 10 Heparin resistance of cleared chylomicron cholesteryl ester as a function of time. Isolated hearts after washing were perfused with medium containing cholesterol-rich chylomicrons (6.0% cholesteryl ester) (5 μg cholesteryl ester/ml) for 2 min, then perfused without recirculation for the period indicated (perfusion period) with Krebs-bicarbonate buffer. At the end of this period, the hearts were washed with heparin-containing medium as described in Fig. 8, for 5 min, and then the hearts were homogenized, extracted with chloroform-methanol, and heart lipids fractionated by thin-layer chromatography. The cholesteryl ester content of the postheparin perfusate was determined by the same means from samples extracted with chloroform and methanol. Percent heparin releasable cholesteryl ester radioactivity is expressed in terms of cpm released by heparin within 5 min/heparin-releasable cpm plus heart cholesteryl ester radioactivity. Percent hydrolysis at each period is expressed as cpm in heart unesterified fatty acid/total heart radioactivity. Each point represents the mean of duplicate determinations.

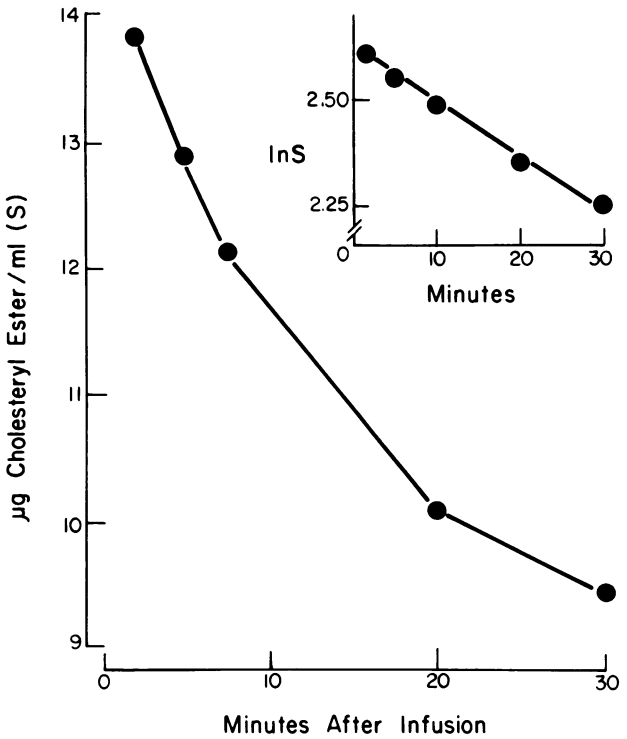

FIGURE 11 Clearance of chylomicron cholesteryl [1-^{14}C]-oleate (7.8% wt/vol) from the plasma compartment of the functionally hepatectomized rat. After injection of the bolus of chylomicrons (3.5 mg triglyceride) into the femoral vein, blood samples were taken at the times indicated through a carotid artery cannula kept patent with saline (15), the plasma obtained by centrifugation and duplicate 0.1-ml samples analyzed by liquid scintillation counting after thin-layer chromatography for cholesteryl ester radioactivity. Values were corrected for dilution by reference to a plasma protein content of 60 mg/ml. First-order removal was maintained in this preparation for at least 2 h of recirculation. S, chylomicron cholesteryl ester concentrations.

with cholesterol-poor chylomicrons are compatible with this process (Fig. 8), and also with previous chemical determinations of remnant cholesteryl ester, given its low content in chylomicrons from triglyceride-fed animals.

Cholesterol may be carried in rat lymph chylomicrons at a rate of up to 0.6 mg/h, which may be associated with up to 20 mg/h of triglyceride (22). Thus, lymphatic particles under these conditions will contain an average cholesteryl ester/triglyceride weight ratio of 0.03. Somewhat higher ratios have been reported for postprandial particles in man (8). Available evidence suggests that chylomicrons are catabolized by membrane-supported LPL until about 80% of their initial triglyceride content has been hydrolyzed, at which point they rapidly cease to be effective substrates for the enzyme (1), and the remnants are removed by the liver (6, 7). These results indicate that the cholesteryl ester content of dietary particles catabolized by the LPL pathway in both species may be on occasion at least as high as 10% by weight. Because the early stages of lipolysis are without effect on the kinetic properties of removal (1) and because the reaction rate of LPL in the heart with control and cholesterol-enriched particles is the same (9), the present study has used chylomicrons with cholesteryl ester contents of 1–10% weight to determine the parameters of cholesteryl ester metabolism. The properties of the pathway by which the heart receives cholesteryl ester from chylomicrons are evidently distinct from those reported for the LDL-receptor site in aortic endothelial cells (23), smooth muscle cells (24), and fibroblasts (20). They are also distinct from those of the LPL site for triglyceride hydrolysis. First, the pathway appears to be nonsaturable, at least at physiologically significant levels of chylomicrons. This was shown by the absence of effect of unlabeled chylomicrons in 40-fold excess on the clearance of labeled chylomicron cholesteryl ester. The same result is indicated from the concentration dependence of clearance of cholesteryl ester and triglyceride (Fig. 7) which showed that although cholesteryl ester uptake was independent of concentration, triglyceride hydrolysis was saturable. Because the chylomicrons used in this study have 6% wt/vol of cholesteryl ester, if triglyceride and cholesteryl ester were cleared by a common mechanism, then the latter would be saturable with an apparent K_m of 2.5 μg cholesteryl ester/ml, corresponding to 0.05 mM triglyceride, the value found for chylomicron triglyceride (Fig. 7). Uptake of cholesteryl ester was proportional to chylomicron cholesteryl ester content, for both cholesterol-rich and cholesterol-poor particles. Uptake of cholesteryl ester was decreased about twofold in the presence of 100% plasma protein. Under these conditions, the removal rate was about 0.004 min. This rate may be compared with that found in the hepatectomized and intact rat (0.015 and 0.10/min), which, although emphasizing the role of the liver in clearance, also suggests that the coronary bed may provide a significant fraction of clearing capacity in the extrahepatic tissues.

A second distinguishing feature of the chylomicron cholesteryl ester pathway is its inhibition (95%) by very low levels of heparin (10 μg/ml). This level is at least two orders of magnitude less than that reported for the LDL receptor, as found in fibroblast (25) and in aortic endothelium (23). Perfusion of heparin was associated with release of cholesteryl ester and LPL, perhaps indicating the release of intact chylomicrons with LPL into the medium. However, the cholesteryl ester cleared from the medium was rapidly removed from its binding site, because it rapidly became resistant to release by heparin (Fig. 10) and was slowly hydro-

lyzed in the tissue. The mechanism now described does not involve endocytosis or pinocytosis of the chylomicron because chylomicron protein was retained in the medium, whereas cholesteryl ester was cleared into the vascular bed.

The chylomicron cholesteryl ester pathway appears to be of considerable potential importance, relative to other pathways, in the supply of plasma cholesteryl ester to the vascular bed, particularly as other data (23) indicate that the LDL receptor pathway is in large part inoperative in this tissue. For particles containing 6% wt/vol cholesteryl ester, circulating at a concentration of 0.1 mM chylomicron triglyceride (5 μg/ml chylomicron cholesteryl ester), the uptake by the heart alone (in the presence of plasma) will be $0.003 \times 15 \times 5$ μg/min for a plasma volume of 15 ml (4.5% body wt) or 13.5 μg/h. The surface area of endothelium in the heart is about 400 cm^2/g tissue (26) and on this basis of per cell surface area (27), the endothelial cells of the heart have a protein content of about 15 mg/g tissue. Bovine aortic endothelial cells in the presence of saturating concentrations of LDL clear cholesteryl ester by the sum of specific receptor and nonspecific pathways at a rate of 60–80 ng cholesteryl ester/mg cell protein (23), and therefore the rate of uptake into the heart at saturation, i.e., the calculated rate of uptake by total heart endothelium, would be only 1.0 μg/h under the same conditions. This calculation, of course, is based on a number of assumptions. First, aortic endothelial cells may not be representative of total heart endothelium, the major part of which is derived from capillaries, although the properties of endothelium from several sites appear similar. Second, the calculation applies only to the endothelium, as is probably correct for chylomicrons, because these are not filtered to the lymph; however, LDL is present in significant concentration in peripheral lymph (28). This will affect the total uptake of LDL by the tissue but not that by the endothelium. Third, the uptake of cholesteryl ester from the remnants generated by chylomicron catabolism may be qualitatively different from that found in intact particles. However, analysis of remnants of cholesterol-enriched chylomicrons (Table I) suggests that these continue to lose cholesteryl ester to the vascular bed. Finally, in the intact animal, as compared to the isolated heart, the liver reduces the circulating half-life of chylomicrons about sixfold (from 0.016 to 0.10); if in the intact animal, as in the isolated heart, the extent of uptake is proportional to circulation time and to cholesteryl ester content, then the rate of clearance by the heart will be reduced in proportion.

On this basis, the liver appears to play the major role in minimizing the uptake of chylomicron cholesteryl ester into the endothelium; the estimates made above suggest that under normal conditions, i.e. with functional hepatic uptake of remnant particles, cholesteryl ester uptake from the LDL and chylomicron pathways is still of comparable extent. In the absence of a functional hepatic removal system, the chylomicron pathway appears to assume major importance, on two grounds: the concentration of remnants will rise, and also their cholesteryl ester/triglyceride ratio (because of the continuing action of LPL with this competitive substrate) (1) will increase. The accumulation of remnants, for example by malfunction of the hepatic clearing system, would be, therefore, expected to result in increased uptake of cholesteryl ester into the vascular bed. A specific genetic disease (type III hyperlipoproteinemia, dysbetalipoproteinemia) is characterized by accumulation of chylomicron remnants in the plasma (29) associated with a defect of hepatic removal (30). This disease is characterized by a high incidence of atherosclerotic vascular disease. It appears possible that this is mediated through the activity of the chylomicron cholesteryl ester pathway characterized in this research, secondary to the accumulation of remnant particles. The pathway now described may also provide a direct mechanism by which high levels of dietary cholesterol could be linked to atherogenesis.

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