Chylomicron remnant cholesteryl esters as the major constituent of very low density lipoproteins in plasma of cholesterol-fed rabbits

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Abstract Feeding rabbits 500 mg of cholesterol daily for 4 to 15 days greatly increased the concentration of esterified cholesterol in lipoproteins of $d < 1.006$ g/ml. The origin of hypercholesterolemic very low density lipoproteins was investigated by monitoring the degradation of labeled lymph chylomicrons administered to normal and cholesterol-fed rabbits. Chylomicrons were labeled in vivo by feeding either *1*) [^{3}H]cholesterol and [^{14}C]oleic acid or *2*) [^{14}C]cholesterol and [^{3}H]retinyl acetate. After intravenous injection of labeled chylomicrons to recipient rabbits, [^{14}C]triglyceride hydrolysis was equally rapid in normal and cholesterol-fed animals. Normal rabbits rapidly removed from plasma both labeled cholesteryl and retinyl esters, whereas cholesterol-fed rabbits retained nearly 50% of doubly labeled remnants in plasma 25 min after chylomicron injection.

Ultracentrifugal separation of plasma into subfractions of very low density lipoproteins showed that chylomicron remnants in cholesterol-fed animals are found among all subclasses of very low density lipoproteins. Analysis of cholesteryl ester specific activity–time curves for the very low density lipoproteins subfraction from hypercholesterolemic plasma showed that nearly all esterified cholesterol in large very low density lipoproteins and approximately 30% of esterified cholesterol in small very low density lipoproteins was derived from chylomicron degradation. Apparently, nearly two-thirds of the esterified cholesterol in total very low density lipoproteins from moderately hypercholesterolemic rabbits is of dietary origin.

Supplementary key words retinyl esters · hypercholesterolemia · hepatic chylomicron remnant removal

The metabolic origins and interrelationships among plasma lipoproteins are presently the subject of intense interest (for review, see ref. 1). It has been known for many years that feeding cholesterol-enriched diets to rabbits rapidly results in increased concentrations of plasma VLDL, which have a large proportion of esterified cholesterol and relatively little triglyceride (2, 3). However, the metabolic origin of VLDL in the cholesterol-fed rabbit is not known. At least two sources of hypercholesterolemic VLDL should be considered. These particles could originate from liver, or from the degradation of triglyceride-rich intestinal lipoproteins. A third possibility, that most of the VLDL cholesteryl ester originates in plasma by the action of lecithin:cholesterol acyltransferase, seems unlikely (4). Noting that hypercholesterolemic VLDL are larger than normal VLDL and have a different apolipoprotein distribution, Shore, Shore, and Hart (5) concluded that hypercholesterolemic VLDL are not the degradation products of normal triglyceride-rich plasma VLDL. They suggested that hypercholesterolemic VLDL are likely to be secreted from liver with a greater cholesteryl ester content than normal VLDL. An intestinal origin for esterified cholesterol in hypercholesterolemic VLDL was suggested to us by the high cholesteryl ester to triglyceride ratio and by the large size of these particles. Increases in chylomicron esterified cholesterol relative to triglyceride are known to result from partial degradation of chylomicrons by extrahepatic tissues in vivo (6,7) or from exposure of chylomicrons to lipoprotein lipase in vitro (8). Hypercholesterolemic VLDL could therefore be partially degraded intestinal chylomicrons, known as chylomicron remnants. In normal rats, the circulation time for chylomicron remnants in plasma appears to be very short. Redgrave (7), who prepared remnant-rich plasma in hepatectomized rats and injected it into plasma of normal recipients, observed that chylomicron remnant cholesteryl esters have a half-life of 1–2 min in plasma, and are subsequently found in liver. The effect that feeding a cholesterol-

Abbreviation: VLDL, very low density lipoprotein(s).

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enriched diet might have on chylomicron remnant removal has not been studied. We report here on the degradation of intestinal chylomicrons in unanesthetized rabbits that have been fed either normal or cholesterol-supplemented diets.

Identification of chylomicron remnants formed under physiological conditions is complicated for two reasons. First, Mjøs et al. (6) have found that remnants prepared in 4-aminopyrozolopyrimidine-treated, supradiaphragmatic rats have size and density distributions overlapping those of endogenous VLDL, so separation of chylomicron remnants from endogenous particles by ultracentrifugation or gel filtration chromatography is not possible. Second, removal of isotopically labeled chylomicron cholesteryl ester from plasma may be incorrectly interpreted if labeled cholesteryl esters are recirculated in endogenous VLDL.

To study the metabolic origin of cholesteryl esters in hypercholesterolemic VLDL of intact, unanesthetized rabbits, we have employed a double labeling technique that discriminates lipoproteins containing cholesteryl esters of dietary origin from hepatogenous lipoproteins. This technique utilizes labeled esters of retinol to trace particles of intestinal origin. Esters of retinol (9) and cholesterol (10) are cotransported in intestinal chylomicrons. After hydrolysis of most chylomicron triglyceride, cholesteryl and retinyl esters are subsequently found in liver. Whereas labeled esterified cholesterol may be recirculated in endogenous VLDL, chylomicron retinyl esters reappear in plasma as free retinol, complexed to retinol-binding protein with a density greater than that of the plasma lipoproteins (11). Thus, only VLDL of intestinal origin will contain labeled esterified retinol. We have utilized this qualitative technique to show that chylomicron remnants circulate for prolonged times in the plasma of rabbits made hypercholesterolemic by short-term cholesterol feeding. We have also monitored the conversion of lymph chylomicron cholesteryl esters to plasma VLDL cholesteryl esters in order to estimate the proportions of VLDL cholesteryl esters that are of dietary and of endogenous origins.

MATERIALS AND METHODS

Isotopes and solvents

[1,2-$^3H_2$]Cholesterol and [4-^{14}C]cholesterol were obtained from Amersham/Searle Corp. (Arlington Hts., IL) and were purified by thin-layer chromatography on silica gel H plates with hexane–diethylether 50:50 (v/v), so that greater than 95% of the labeled sterol comigrated with dibromide purified cholesterol. [1-^{14}C]Oleic acid was obtained from Dhom Products (North Hollywood, CA). It was certified to be greater than 99% pure. [1-^{14}C]Oleyl cholesterol was obtained from Amersham/Searle Corp. and was purified for use as a recovery standard by thin-layer chromatography on silica gel H with a solvent system of hexane–diethylether 80:20 (v/v). [11,12-$^3H_2$]Retinyl acetate (320 μCi/mg) was the gift of Hoffman-LaRoche, Inc., (Nutley, NJ). More than 90% of the [^{3}H]retinyl acetate comigrated with unlabeled retinyl acetate (U.S.P. vitamin A reference standard, U.S. Pharmacopeial Convention, Inc., New York, NY), as measured by absorbance at 330 nm, when mixtures were chromatographed on aluminum oxide columns as described below, or with the solvent systems of Huang and Goodman (12). Calibrated [^{3}H]- and [^{14}C]toluene were obtained from New England Nuclear (Boston, MA) and used as dpm standards. Solvents were redistilled before use or were of analytical grade. Solvents used for extraction and chromatography of vitamin A compounds contained 5 μg/ml of butylated hydroxytoluene (Nutritional Biochemicals Corp., Cleveland, OH) and were used under red or very dim light.

Animals and diets

All rabbits were females of the New Zealand white strain obtained from Becken's Research Animal Farm (Sanborn, NY). Rabbits weighed 2.5–4 kg and were individually housed. Cholesterol-fed rabbits were fed 100 g daily of Purina Laboratory Rabbit Chow (Ralston Purina, St. Louis, MO), supplemented with 500 gm of cholesterol (U.S.P., Nutritional Biochemicals, Cleveland, OH) and 2.7 g of oil (Wesson Oil, Hunt Wesson, Fullerton, CA). Control animals were fed chow supplemented only with oil. Since this chow contained approximately 2.2% fat, the total fat content of the diet was 5%.

Estimation of plasma volume

Plasma volumes were calculated as 3.28% of body weight. This average value was determined by the Evans' Blue dye dilution technique (13) in a separate group of control rabbits (n = 12, SD = ±0.31% of body weight).

Cannulation and preparation of labeled chylomicrons

Cannulation of the thoracic duct was performed as described by Rudel, Morris, and Felts (14) except that the left kidney was not removed, and an intraduodenal tube (Silastic, 0.03 in ID, 0.065 in OD, Dow Chemical Co., Midland, MI) for infusion of saline or the isotopic dose was placed 3–5 cm from

the pylorus. When rabbits had recovered for 1–4 days and were eating, a labeled dose (below) was infused. In two experiments, absorbing lymph donors, one cholesterol-fed and one control, were given an infusion containing approximately 250 μCi of [^{3}H]-cholesterol and 175 μCi of [^{14}C]oleic acid dissolved in 2 ml of Wesson oil. In four experiments, donors fed cholesterol were given an intraduodenal dose of 100–200 μCi of [^{14}C]cholesterol and 0.5–1 mCi of [^{3}H]retinyl acetate. The isotopic compounds were dried under N_2 and dissolved in 0.2 ml of 4% Tween 20 (I.C.N. Pharmaceuticals, Cleveland, OH) in 95% ethanol essentially as described by Whereat and Staple (15). After evaporation of ethanol, 2 ml of saline was added and solubilized radioisotopic compounds were infused over a 2–4 hr period through the duodenal cannula. One cholesterol-fed donor received 1 mCi of [^{3}H]cholesterol only, solubilized in Tween. Lymph was collected on ice. Ethylenediaminetetraacetic acid (EDTA), 0.1 mg/ml of lymph, was added to each collection as a preservative (16); chylomicrons for injection were prepared within a day of lymph collection.

Lipoprotein preparations

For preparation of thoracic duct lymph chylomicrons, lymph was brought to room temperature, defibrinated, and filtered through gauze (17). Lymph was centrifuged at 10°C in the 40 rotor (Beckman Instrument Co., Palo Alto, CA) by the procedure of Gustafson, Alaupovic, and Furman (18) in which chylomicrons from large volumes of lymph may be prepared quickly. Chylomicrons of $S_f > 400$ were diluted twofold with buffer (18) and then washed and concentrated once by centrifugation as described above.

Classes of plasma lipoproteins were separated either by sequential preparative ultracentrifugation at solvent densities of 1.006, 1.019, and 1.063 (19) or, when the purpose was to separate subclasses of very low density lipoproteins, by a single centrifugation in a discontinuous salt gradient. The procedure of Minari and Zilversmit (17) was used, with modification for the Beckman SW 41 rotor: 4.2 ml of diluted sample at d 1.063 was pipeted into a cellulose nitrate tube and overlayered with 2 ml each of salt solutions of d 1.041 and d 1.019, and 3.8 ml of salt solution of d 1.006. Centrifugation conditions were chosen with reference to the nomogram of Dole and Hamlin (20) to collect, by sequential tube slicing, four lipoprotein fractions designated in the text as VLDL-1, VLDL-2, VLDL-3, and $S_f < 20$ lipoproteins. For lipoproteins of a density of 0.97 g/ml, the diameters of these fractions, calculated from the formula of Dole and Hamlin, are ≥75 nm, 49–75 nm, 23–49 nm, and <23 nm, respectively. Radioactivity recovered in the sum of VLDL-1, -2, and -3 equalled 97.5% ± 8.5% (mean ± SD) of radioactivity in total VLDL of d < 1.006 in nine paired comparisons.

Administration of chylomicron dose

Recipient rabbits were fed 2–3 hr before injection of the chylomicron dose, which contained 17–23 mg of triglyceride per kg of body weight. The dose was injected into a marginal ear vein after local anesthesia with Novacain (procaine hydrochloride, 2%, Winthrop Laboratories, New York, NY) given with a Dermo-Jet (Robbins Instrument Co., Chatham, NJ). Serial blood samples were collected with EDTA (0.4M, 0.010 ml/ml blood) from the opposite ear and chilled immediately. After 4 min to 2 hr, rabbits were anesthetized with sodium pentobarbital given intravenously, and livers were removed and frozen for subsequent analysis of radioactivity. In one experiment (Fig. 3), livers were perfused in situ with 80 ml of cold saline.

Lipid extractions, chromatography, and isotope measurement

Neutral lipids in aqueous samples (plasma, chylomicrons, or lipoprotein fractions) were partitioned into hexane from 40% ethanol (21). This extraction was found to be quantitative for triglycerides, and for free and esterified cholesterol and retinol. Aliquots of hexane were taken for column chromatographic separation of free and esterified forms of retinol and cholesterol on alumina (2 g, Woelm neutral or W200 neutral aluminum oxide, ICN Pharmaceuticals, Cleveland, OH) deactivated to grade III (21). Aliquots of hexane extracts were evaporated under N_2 and applied to alumina columns in a one-ml volume of hexane followed by a one-ml rinse with hexane. Ten ml of hexane–diethylether 95:5 eluted both retinyl and cholesteryl esters and, subsequently, 15 ml of diethylether eluted both free cholesterol and retinol. Recoveries reported in footnote *b* to Table 1 were typical of all separations. Eluates collected in counting vials were dried and dissolved in toluene-based liquid scintillation solution. No quenching was detected. Efficiency and overlap corrections were made by reference to [^{3}H]- and [^{14}C]toluene dpm standards. Since batches of deactivated alumina could differ slightly, each batch was tested with TLC-purified [^{14}C]cholesteryl oleate and [^{3}H]cholesterol (approximately 2 mg each) immediately before use to assure complete separation of esterified and free cholesterol and recoveries of greater than 95% of applied test sterols. Absorption measure-

ments (330 nm) showed that 98% of unlabeled retinyl palmitate (Nutritional Biochemicals, Cleveland, OH), purified on alumina, rechromatographed in the fraction eluted with hexane–diethylether 95:5 (v/v).

For determination of cholesteryl ester and triglyceride specific activities, hexane extracts were evaporated and separated on thin-layer plates of silica gel H with a solvent system of hexane–diethylether 80:20 (v/v). Esters were eluted from silica gel scrapings with chloroform–methanol 9:1 (v/v) and assayed as described below.

Chylomicron or tissue total lipids were extracted with chloroform–methanol 2:1 (v/v), according to Folch, Lees, and Sloane Stanley (22). Chylomicron extracts were washed and separated into neutral lipids and phospholipids on silicic acid columns (17); neutral lipids were further separated by thin-layer chromatography. Aliquots of chloroform–methanol extracts of liver, lung, and spleen were evaporated and dissolved in 0.25 ml MeOH and 10 ml toluene scintillation fluid. Corrections for quench and overlap were made after addition of internal standards of [^{14}C]- and [^{3}H]cholesterol.

Chemical analyses

Cholesterol, triglycerides, and phospholipid phosphorus were measured by standard procedures previously described (23).

Statistics

Results reported are means ± 1 SD. Student's *t* test for paired or independent samples was conducted according to Snedecor and Cochran (24).

RESULTS

Plasma lipoprotein changes in response to short-term cholesterol feeding

Control rabbit plasma cholesterol concentrations equalled 35–100 mg/dl; generally less than 10% of plasma cholesterol was contained in VLDL. When the diet was supplemented with 500 mg of cholesterol daily for 2 weeks, plasma cholesterol rose to 605–1119 mg/dl in five rabbits. In three of these animals, total cholesterol was distributed among the four major lipoprotein classes as follows: VLDL ($d < 1.006$) 57 ± 12%, intermediate density ($1.006 < d < 1.019$) 16 ± 6.4%, low density ($1.019 < d < 1.063$) 20 ± 8.7%, high density ($d > 1.063$) 9.1 ± 3.6%. The elevation of VLDL cholesterol was associated with increases in the molar ratio of esterified to free cholesterol from 1.7 ± 0.1 in control VLDL to 4.4 ± 0.3 in hypercholesterolemic VLDL. The esterified cholesterol to triglyceride ratio increased from 0.36 ± 0.09 in control VLDL to 22 ± 4.3 in VLDL from cholesterol-fed rabbits.

Fig. 1 shows that cholesterol in VLDL ($d < 1.006$) increased within 6 hr after feeding the first cholesterol-containing meal to normal rabbits. This increase in VLDL preceded any change in the concentration of cholesterol carried in lipoproteins of $d > 1.006$, which is primarily low density lipoprotein. Very low density lipoprotein cholesterol concentrations showed cyclical fluctuations. Maximum values were observed 9–12 hr after feeding, with no further increase, or in some rabbits a decrease, in VLDL cholesterol concentrations in postabsorptive samples. The concentration of cholesterol in lipoproteins of $d > 1.006$ began to increase later than VLDL cholesterol and did not decrease in the postabsorptive state (24 and 48 hr). While these chemical measurements cannot establish the origin of VLDL cholesterol, the results are consistent with rapid accumulation of dietary cholesteryl esters in remnants of $d < 1.006$, and subsequent conversion of remnants or endogenous VLDL to lipoproteins of higher densities.

Composition of the labeled chylomicron dose

When labeled chylomicrons of $S_f > 400$ were isolated from thoracic duct lymph of rabbits actively absorbing chow diets containing 5% fat and 0.5% cholesterol, lymph chylomicrons contained as much as 7% cholesterol, of which an average of 72% was esterified **(Table 1).** More than 75% of labeled chylomicron cholesterol and more than 90% of labeled chylomicron retinol were esterified. Thus, the metabolism of chylomicron cholesteryl esters could be studied with little interference due to esterification of labeled free cholesterol in plasma.

Nonexchangeability of chylomicron [^{3}H]retinyl esters

The nonexchangeability of chylomicron esters with plasma lipoproteins was tested by incubating doubly labeled chylomicrons with either normal or hypercholesterolemic rabbit plasma. The proportions mixed were chosen to equal the ratio of injected chylomicron mass to plasma volume in the metabolic studies below (0.6–0.7 mg chylomicron triglyceride per ml plasma). After 0–60 min of incubation at 37°C, the mixture was centrifuged to separate chylomicrons (VLDL-1), VLDL-2, VLDL-3, and $S_f < 20$ lipoproteins plus plasma proteins. After incubation, there was no observable transfer of either labeled cholesteryl or retinyl esters to VLDL-2 or VLDL-3. In the case of [^{3}H]retinyl esters, recovery

of label in lipoproteins of $S_f < 20$ was also very small. However, in the case of [^{14}C]cholesteryl esters, some transfer to the $S_f < 20$ fraction was observed. **Table 2** indicates that, after 25 min, 14% of chylomicron cholesteryl esters were recovered among lipoproteins or proteins of $S_f < 20$. Had chylomicrons been labeled only with esterified cholesterol, it would not have been possible to distinguish transfer of cholesteryl esters from sticking, or coalescence, of chylomicrons to plasma lipoproteins. However, because [^{3}H]retinyl esters were not found among $S_f < 20$ lipoproteins, whereas a small proportion of [^{14}C]cholesteryl esters were recovered there, it appears that some chylomicron cholesteryl esters are exchangeable, whereas retinyl esters constitute a nonexchangeable marker for chylomicrons.

Disappearance of chylomicron triglyceride

[^{14}C]Oleate-labeled triglyceride in lymph chylomicrons was removed from plasma of recipient rabbits at constant fractional rates for at least 15 min. The half-life for removal of triglyceride from chylomicrons prepared from lymph of a cholesterol-fed donor was 7.9 ± 0.65 min in control recipients and 7.5 ± 0.40 min in recipient rabbits fed 500 mg cholesterol for 4 days **(Table 3).** When chylomicrons from a normal donor fed only chow plus fat were administered, half-times were 4.9 ± 1.2 min and 4.4 ± 0.85 min in cholesterol-fed and control rabbits, respectively. Whereas cholesterol-fed and normal rabbits hydrolyzed chylomicron triglyceride from the same source at equal fractional rates, and because both the plasma triglyceride pool size and the dietary fat intake were the same in rabbits fed cholesterol for 4 days and in normal rabbits, we have concluded that short-term cholesterol feeding produced no decrease in the rate of degradation of chylomicrons by lipoprotein lipase. The shorter half-times for triglyceride hydrolysis observed when normal chylomicrons were injected could be related to structural or chemical differences, but might also be due in part to the slightly lower mass of the injected chylomicrons (footnotes to Table 3).

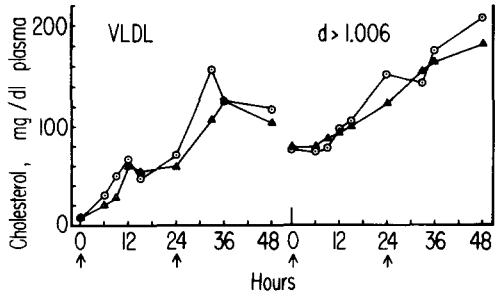

FIG. 1. Changes in rabbit plasma VLDL and d > 1.006 lipoprotein cholesterol concentrations upon initiation of cholesterol feeding. Rabbits previously fed cholesterol-free chow diets were fed 100 g of chow supplemented with 500 mg of cholesterol and 2.7 g of fat at 0 hr and at 24 hr (↑). Nearly all food was consumed within 3 hr. Data for two representative rabbits out of six rabbits are illustrated. Control rabbits (not shown) were fed chow plus fat only and had nearly constant concentrations of lipoprotein cholesterol throughout the day.

Disappearance of chylomicron retinyl and cholesteryl esters from plasma

After injection of cholesterol-rich chylomicrons labeled with [^{3}H]retinyl esters and [^{14}C]cholesteryl esters, both labeled esters disappeared rapidly from the plasma of normal rabbits **(Fig. 2A).** In hypercholesterolemic rabbits, labeled esters of both retinol and cholesterol circulated in plasma for much longer times, indicating the prolonged presence in plasma of lipoproteins of intestinal origin (Fig. 2B). Differences in the clearance of labeled chylomicron cholesteryl esters from plasma were found to be related to the diets fed to recipient animals, rather than to the source in injected chylomicrons. When cholesteryl ester-labeled lymph chylomicrons from a normal donor were administered to rabbits fed either normal or cholesterol-supplemented diets, results (not shown) were identical to those illustrated in Fig. 2. Because chylomicrons from cholesterol-fed donors

TABLE 1. Lipid composition of labeled chylomicrons prepared for injection[a]

Percent of Chylomicron Lipid Mass				Percent of Chylomicron Radioactivity[b]			
				^{3}H		^{14}C	
TG[c]	TC[c]	(% esterified)	PL[c,d]	esterified retinol	free retinol	esterified cholesterol	free cholesterol
87 ± 2.7	5.7 ± 1.9	72 ± 9.7	7.7 ± 0.91	98 ± 0.56	2.0 ± 0.55	85 ± 7.5	15 ± 7.4

[a] Results are means ± SD for four chylomicron preparations from four cholesterol-fed donor rabbits.
[b] The recovery of radioactivity in eluates from alumina columns was 96% ± 3.7% for ^{3}H and 96% ± 3.7% for ^{14}C in eight extracts of chylomicron lipids.
[c] Abbreviations are: TG, triglyceride; TC, total cholesterol; PL, phospholipid.
[d] Phospholipid mass calculated as phospholipid phosphorus × 25.

TABLE 2. Recovery of labeled chylomicron esters among $S_f < 20$ lipoproteins after in vitro incubation with plasma[a]

Minutes of Incubation at 37°C[b]	[^{3}H]Retinyl Esters[c]	[^{14}C]Cholesteryl Esters[c]
0[d] (n = 3)	1.8 ± 0.4	1.0 ± 0.2
5 (n = 1)	1.7	3.5
25 (n = 4)	3.0 ± 0.4	14.4 ± 3.3
60 (n = 1)	4.2	17.3

[a] Results of 0 min and 25 min incubations with normal or hypercholesterolemic rabbit plasma were identical, therefore results for both types of plasma are combined here.
[b] One ml of plasma and 0.6–0.7 mg of chylomicron triglyceride were mixed. Incubations were stopped by chilling on ice and samples were ultracentrifuged immediately.
[c] Percent of activity in labeled chylomicrons recovered in the $S_f < 20$ fraction.
[d] Mixed and held at 0°C until centrifuged.

contained more labeled cholesterol and a greater fraction of esterified labeled cholesterol than chylomicrons collected from control donors, chylomicrons from cholesterol-fed donors were used in subsequent studies.

Evidence that labeled cholesteryl esters in plasma are undegraded intestinal products comes from experiments in which chylomicron cholesteryl esters were doubly labeled with [^{3}H]cholesterol and [^{14}C]-oleic acid. The ratio of ^{14}C to ^{3}H in esterified cholesterol was 0.021 in the injected dose and 0.022 ± 0.001 at all times from 2–40 min in plasma cholesteryl esters of both control (n = 3) and cholesterol-fed (n = 3) rabbits.

Uptake of labeled retinyl and cholesteryl esters by liver

The removal of labeled chylomicrons from plasma and the hepatic uptake of label were compared in control rabbits and in rabbits fed cholesterol for 4 days or for 15 days. Individual animals were killed 4–50 min after intravenous administration of chylomicrons labeled with [^{3}H]retinyl esters and [^{14}C]cholesteryl esters. Clearance of both labeled esters was nearly identical, and results are reported only for [^{3}H]-retinyl ester activity. **Fig. 3A** illustrates that removal of tritium (more than 90% of which was in esterified retinol) from plasma was rapid in both control and cholesterol-fed rabbits for approximately 10 min after injection. In control animals, plasma radioactivity continued to decrease while retinyl ester activity in plasma of cholesterol-fed rabbits remained nearly constant after 10 min. Fig. 3B shows that the greater disappearance of radioactivity from control plasma is accounted for by a greater uptake into liver.

Panel C, Fig. 3, which illustrates the sum of tritium activity in liver plus plasma, indicates that less than 50% of the injected dose can be accounted for in the two tissues at 10 min. During the first 10 min after chylomicron injection, when more than 55% of chylomicron triglyceride was removed (Table 3), less than 10% of the injected retinyl ester radioactivity was found in liver (Fig. 3B). Between 30 and 50 min after administration of the labeled chylomicron dose, the sum of ^{3}H activity in liver plus plasma equaled only 55–72% of the dose in all dietary groups. Tritium in spleen and lung equaled only 0.5 ± 0.6% and 0.7 ± 0.4% (n = 16), respectively, of radioactivity in the injected dose. The location of the remaining radioactivity has not been established.

TABLE 3. Half-times of disappearance of lymph chylomicron [^{14}C]triglyceride from plasma of absorptive rabbits[a]

	Cholesterol-Rich Chylomicrons[b]	Normal Chylomicrons[c]
Cholesterol-fed[d] recipients	7.9 ± 0.65 min (n = 3)	4.9 ± 1.2 min (n = 3)
Control[d] recipients	7.5 ± 0.40 min (n = 3)	4.4 ± 0.85 min (n = 3)

[a] Slopes were log-linear for at least 15 min. The initial slope was used to calculate chylomicron triglyceride half-lives.
[b] Each dose contained 55–59 mg of chylomicron triglyceride.
[c] Each dose contained 44 mg of chylomicron triglyceride.
[d] Average plasma TG concentration was 107 mg/dl for six cholesterol-fed recipients and 115 mg/dl for six control recipients.

Remnants in lipoprotein subfractions

The ultracentrifugal characteristics of retinyl ester-labeled remnants were investigated to determine if these particles have flotation rates characteristic of VLDL. **Fig. 4** shows the retinyl ester radioactivity in VLDL subfractions and $S_f < 20$ lipoproteins prepared by discontinuous gradient ultracentrifugation of plasma. Control rabbits removed more than 95% of injected [^{3}H]retinyl esters from VLDL-1 within 25

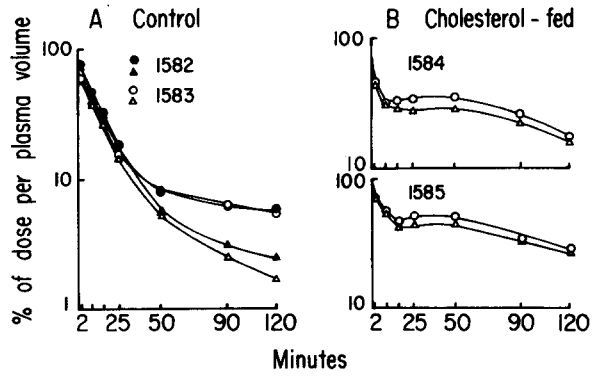

Fig. 2. Disappearance of chylomicron [^{3}H]retinyl esters (△▲) and [^{14}C]cholesteryl esters (○●) from plasma. Recipients were either absorptive control rabbits (A) or absorptive rabbits fed cholesteryl-supplemented chow for 4 days. Labeled chylomicrons used in both groups of recipients were prepared from lymph of a cholesterol-fed donor rabbit.

min. Between 2 and 30 min, labeled retinyl and cholesteryl esters appeared in, and were rapidly removed from, both VLDL-2 and VLDL-3. Thus, formation of chylomicron remnants having flotation characteristics of both large and small VLDL is part of the normal degradation process in the rabbit; these particles circulate for only a short time in control animals. After 25 min, less than 11% of the injected esterified retinol remained in all very

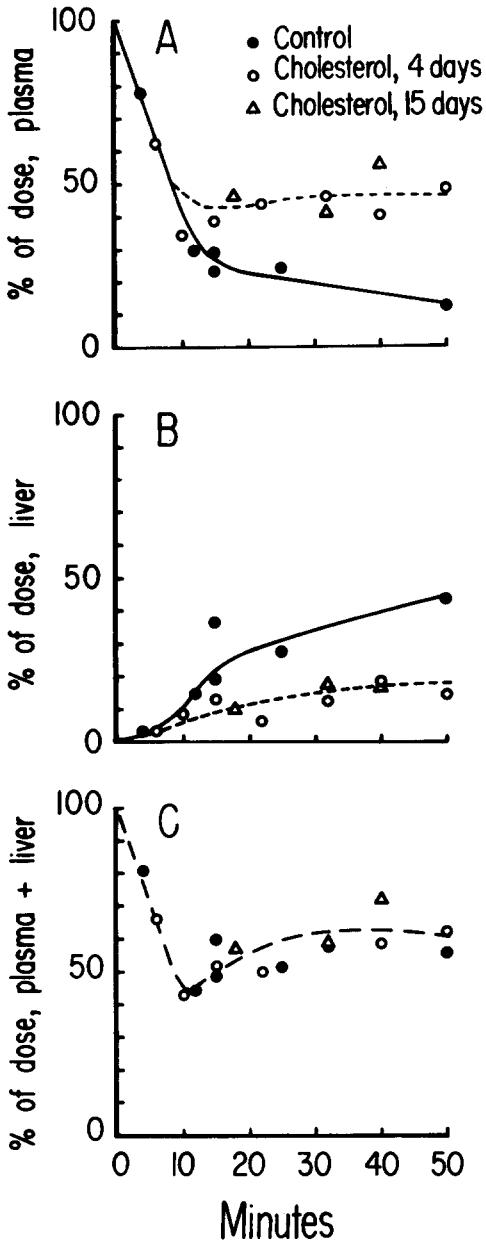

Fig. 3. [^{3}H]Retinyl ester plus retinol activity in plasma (A), liver (B), and the sum of both tissues (C). Rabbits were fed control diet (●), or cholesterol supplemented diet for 4 days (○) or 15 days (△). The average plasma total cholesterol concentrations in animals of these three groups were 54, 192, and 658 mg/dl plasma, respectively.

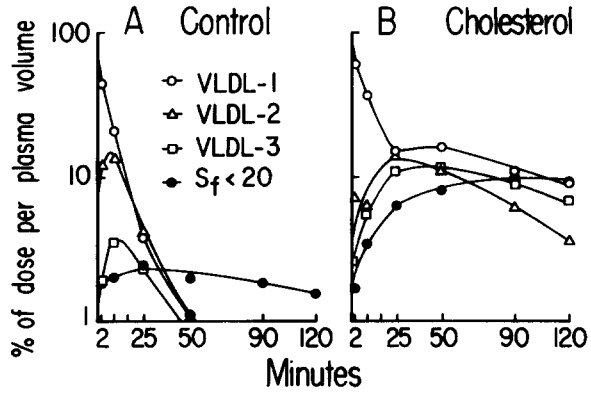

Fig. 4. [^{3}H]Retinyl esters in plasma lipoprotein fractions of normal rabbits and rabbits fed cholesterol for 4 days. Lipoproteins were isolated by discontinuous gradient centrifugation as described in Methods. Results for [^{14}C]cholesteryl esters were nearly identical to those for retinyl esters in VLDL-1, VLDL-2, and VLDL-3. In $S_f < 20$ lipoproteins, cholesteryl ester activity exceeded retinyl ester activity by 2.7–6.6% of the injected dose per plasma volume at each time point.

low density subfractions, while less than 3% was found in lipoproteins of $S_f < 20$. Equivalent results were found in other experiments where total VLDL ($d < 1.006$) was isolated from three normal rabbits and found to contain 12 ± 7.3% of chylomicron retinyl esters after 25 min. In comparison, three hypercholesterolemic rabbits (337–357 mg cholesterol/dl plasma) retained 30 ± 10% of chylomicron retinyl esters in $d < 1.006$ lipoproteins and 10 ± 3.2% in lipoproteins of $d > 1.006$. As shown in Fig. 4B, all subfractions of hypercholesterolemic VLDL retained labeled retinyl esters. Thus, chylomicron remnants in cholesterol-fed rabbits have a broad range of sizes; including particles (VLDL-1) calculated to be greater than 75 nm in diameter (see Methods).

Estimation of the contribution of chylomicron remnants and endogenous particles to plasma VLDL esterified cholesterol

The precursor–product analysis of Zilversmit, Enteman, and Fishler (25) has been used to estimate the proportion of esterified cholesterol in subfractions of VLDL (product) that is derived from lymph chylomicrons (precursor). If all product is derived from the labeled precursor, the specific activity of the product will be maximal at the same time (t_{max}) that it is equal to the specific activity of its labeled precursor. If, however, there is an additional input of unlabeled material to the product pool, the ratio of product specific activity to precursor specific activity at t_{max} will be equal to the fraction of compound in the product pool that is derived from the labeled precursor (26). When this analysis is applied to formation of remnant cholesteryl esters from

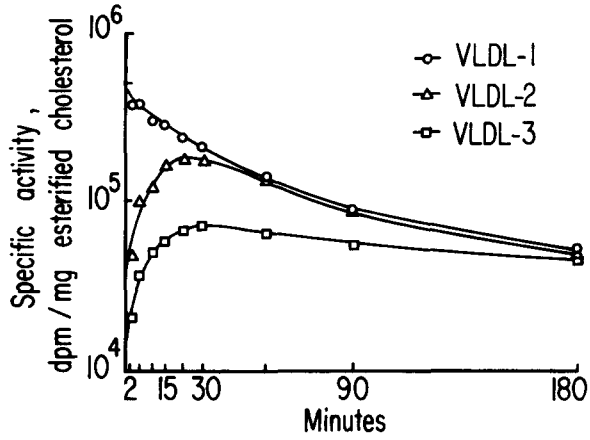

Fig. 5 Specific activity of esterified cholesterol in VLDL subfractions from a hypercholesterolemic rabbit (1626) after intravenous labeling with [^{3}H]cholesteryl ester-labeled chylomicrons.

labeled chylomicron cholesteryl esters and dilution with unlabeled cholesteryl esters in endogenous VLDL, the proportion of remnant esterified cholesterol in a fraction of VLDL may be calculated.

Cholesterol-fed or normal animals were injected with [^{3}H]cholesteryl ester-labeled lymph chylomicrons ($S_f > 400$) and three plasma VLDL subfractions were prepared at various times thereafter. Results from one experiment are illustrated in **Fig. 5.** The specific activity of cholesteryl esters in VLDL-1 defines the precursor specific activity–time curve. In two rabbits fed cholesterol for 4 days, the specific activity of cholesteryl esters in VLDL-2 equaled 100% and 82% of VLDL-1 cholesteryl ester specific activity at t_{max}, indicating that all, or nearly all, cholesteryl esters in this lipoprotein fraction were transported in lipoproteins formed by degradation of VLDL-1.

The specific activity of cholesteryl esters in VLDL-3 should be compared with the cholesteryl ester specific activity of VLDL-1 if these small particles result directly from degradation of $S_f > 400$ lipoproteins. It should be compared with the cholesteryl ester specific activity of VLDL-2 if smaller VLDL are formed sequentially from larger VLDL. The former comparison, (specific activity of VLDL-3 divided by specific activity of VLDL-1), will give smaller values for the proportion of VLDL-3 cholesteryl esters metabolically related to VLDL-1. By this comparison, 30 and 34% of cholesteryl esters in VLDL-3 were derived from VLDL-1, whereas the rest originated from unlabeled sources.

In normal rabbits, the ratio of cholesteryl ester specific activity in VLDL-2 to that in VLDL-1 was nearly as great (0.71) as that in hypercholesterolemic plasma, but the concentration of esterified cholesterol in VLDL-2 was less than 1 mg/dl plasma. In smaller VLDL (VLDL-3) from control rabbit plasma, cholesteryl ester specific activity at t_{max} was less than 10% of VLDL-1 cholesteryl ester specific activity, indicating an endogenous origin for more than 90% of the esterified cholesterol in this fraction of VLDL.

If all esterified cholesterol in plasma VLDL-1 is of intestinal origin, these data indicate that nearly all esterified cholesterol of VLDL-2 and one-third of that in VLDL-3 are contained in partially degraded chylomicrons. However, if unlabeled cholesteryl esters secreted by liver are also transported as very large particles, such as those found in VLDL-1, then the cholesteryl ester specific activity of this fraction would be less than the specific activity of chylomicron cholesteryl esters. In that case the proportion of VLDL-2 and VLDL-3 derived from chylomicrons would have been overestimated. The following evidence suggests that esterified cholesterol in VLDL-1 is related to dietary, rather than hepatic, cholesterol. **Fig. 6** shows the change in cholesterol concentration of hypercholesterolemic plasma, VLDL-1, -2, -3, and $S_f < 20$ lipo-

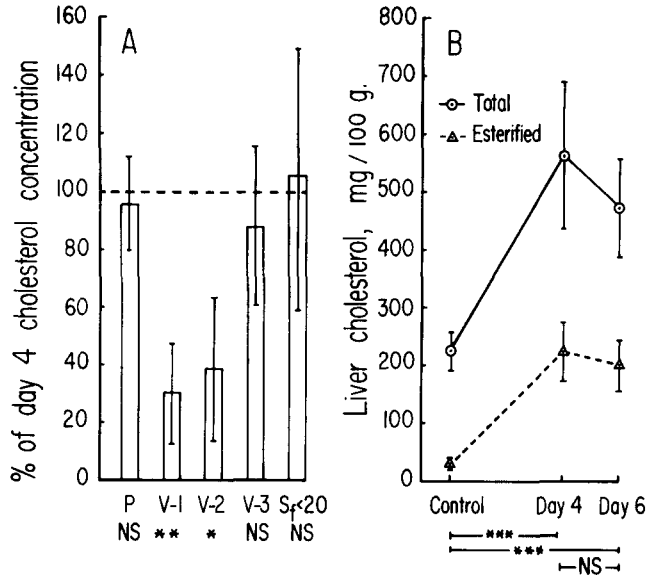

Fig. 6. A: Changes in plasma (P) and lipoprotein cholesterol concentration 2 days after removal of dietary cholesterol. Concentrations measured 3 hr after feeding the fourth cholesterol-supplemented meal (day 4) were defined as 100%. Concentrations measured 3 hr after the second cholesterol-free meal (day 6) are expressed as a percent of day 4 concentrations. V = VLDL. Results were evaluated by Student's t test for paired samples (n = 7; **, $P < 0.025$; *, $P < 0.05$; NS, not significant). The concentrations of plasma total cholesterol on day 4 ranged from 170 to 304 mg/dl plasma. B: Liver cholesterol concentrations in individual rabbits fed control diet (n = 14), cholesterol-supplemented diet for 4 days (n = 11), or cholesterol-supplemented diet for 4 days followed by control chow for 2 days (day 6, n = 4). Student's t test for independent analyses was used to evaluate significance. All differences between day 4 or day 6 and control values were highly significant ($P < 0.001$, ***), while differences between day 4 and day 6 were not significant ($P > 0.2$).

TABLE 4. Proportion of remnant esterified cholesterol in VLDL subfractions and in total VLDL of absorptive rabbits

Animal (Plasma Cholesterol Concentration)	Lipoprotein Fraction	Esterified Cholesterol (A)	Fraction of Esterified Cholesterol Derived from Chylomicrons (B)	Remnant Esterified Cholesterol Concentration (A × B)	Proportion of Remnant Esterified Cholesterol in Total VLDL (A × B)/A
		mg/dl		*mg/dl*	
1623, Cholesterol diet (156 mg/dl)	VLDL-1	24	1.00	24	
	VLDL-2	14	1.00	14	
	VLDL-3	31	0.30	9.3	
	Total[a] VLDL	69		47.3	0.69
1626, Cholesterol diet (237 mg/dl)	VLDL-1	38	1.00	38	
	VLDL-2	24	0.82	20	
	VLDL-3	54	0.34	18	
	Total[a] VLDL	116		76	0.66
Control pool[b], control diet (49 ± 8.6 mg/dl)	VLDL-1	1.3	1.00	1.3	
	VLDL-2	0.9	0.71	0.6	
	VLDL-3	5.7	0.06	0.3	
	Total[a] VLDL	7.9		2.2	0.28

[a] Sum of VLDL-1, -2, and -3.
[b] Plasma samples from three control rabbits were individually fractionated by ultracentrifugation. Equal aliquots of hexane extracts were pooled before TLC and determination of cholesteryl ester specific activity to obtain sufficient mass for analysis.

proteins 2 days after cessation of cholesterol feeding. Although no significant change in whole plasma cholesterol was observed, the concentration of cholesterol in VLDL-1 decreased very significantly. The concentration of cholesterol in VLDL-2 also decreased significantly, consistent with a relationship of cholesterol in this fraction to cholesterol in VLDL-1. During 4 days of cholesterol feeding, the total and esterified cholesterol concentrations in liver increased; both concentrations remained elevated 2 days after cholesterol feeding ended. Thus the concentration of cholesterol in VLDL-1 does not appear related to the level of total or esterified cholesterol in liver, but is related to the input of dietary cholesterol.

It is of interest to calculate the proportion of dietary cholesteryl esters in total VLDL ($d < 1.006$) of the cholesterol-fed rabbit. When all esterified cholesterol in VLDL-1 is considered to be derived from chylomicrons, remnant cholesteryl esters accounted for 66 and 69% of total VLDL cholesteryl esters in two moderately hypercholesterolemic rabbits **(Table 4).**

DISCUSSION

The origin of esterified cholesterol in VLDL of hypercholesterolemic plasma

From these studies we have drawn the conclusion that cholesteryl esters in VLDL of hypercholesterolemic rabbit plasma are primarily intestinal products. After only 4 days of cholesterol feeding, two-thirds of plasma VLDL cholesteryl esters were present in lipoproteins derived from lymph chylomicrons, with the remaining esterified cholesterol originating from other sources. Our calculations assumed that all esterified cholesterol in the largest subfraction of plasma VLDL (VLDL-1) are contained in chylomicrons or large chylomicron remnants. We consider this assumption reasonable in view of the parallelism between the presence of cholesterol in the diet and the concentration of cholesterol in VLDL-1. When cholesterol was added to the diet, the cholesterol concentration in VLDL-1 increased more than 15-fold. When dietary cholesterol was withdrawn for 2 days, the cholesterol concentration in VLDL-1 decreased markedly, although liver cholesterol remained elevated.

Whereas we have concluded that a major proportion of hypercholesterolemic VLDL cholesteryl esters are derived from chylomicrons, a different conclusion has been reached by Shore et al. (5) and Rodriguez et al. (27) based primarily on the large diameters observed for hypercholesterolemic VLDL (5) and on the fatty acid composition of VLDL esterified cholesterol (27). In our studies, characterization of [^{3}H]-retinyl ester-labeled particles by flotation has shown that chylomicron remnants in hypercholesterolemic rabbit plasma have an extremely broad size range, including particles as large as some chylomicrons. The increase of cholesteryl oleate in hypercholesterolemic VLDL (27) would not distinguish between an intestinal and a hepatic origin of cholesteryl esters because cholesterol esterifying enzymes in both intestine (28) and liver (29) preferentially produce cholesteryl oleate. Studies by Rose (4) have been

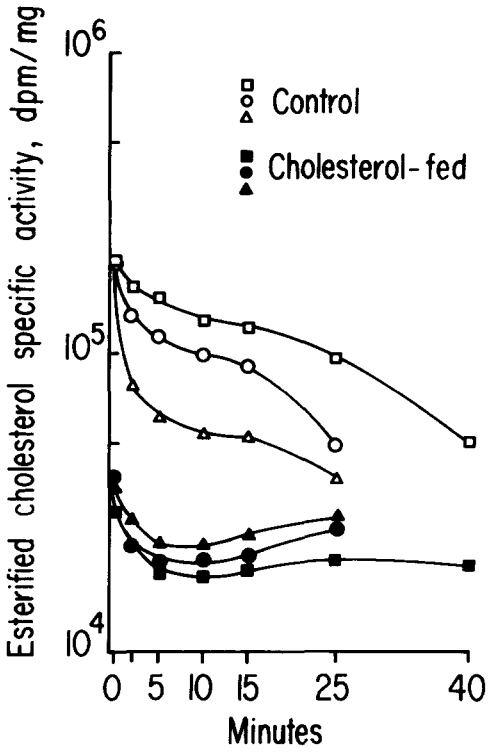

Fig. 7. Cholesteryl ester specific activity in plasma following injection of [^{3}H]cholesteryl ester-labeled chylomicrons. Results for rabbits fed cholesterol for 4 days are represented by filled symbols and for control animals by open symbols.

cited in support of a hepatic origin for hypercholesterolemic VLDL. While Rose recognized that the intestine, as well as liver and lecithin:cholesterol acyltransferase, might contribute to plasma cholesteryl esters, his studies were not directed at a quantitative evaluation of the intestinal contribution.

This work has concentrated on the origin of the apolar "core" lipids of plasma VLDL. From studies in progress, it appears that the arginine-rich apolipoprotein, which Shore et al. (5) have shown to be one of the major apolipoproteins of hypercholesterolemic VLDL, makes up very little of the apolipoprotein component of lymph chylomicrons. It seems possible to us that this component becomes associated with lymph chylomicrons or VLDL during circulation in plasma.[3] If so, it may be inappropriate to speak of the origin of the VLDL particle isolated from plasma. Rather, the possibly different origins of its constituent parts must be considered. From our data, cholesteryl esters in hypercholesterolemic plasma VLDL are largely derived from intestinal chylomicrons.

It may be that the total intestinal contribution to plasma VLDL esterified cholesterol is even larger than the contribution from intestinal chylomicrons. This would be so if a significant portion of dietary cholesterol is transported in lymph VLDL (S_f 20–400), as has been reported by Zilversmit, Courtice, and Fraser (30) and by Rudel et al. (14). However, Redgrave and Dunne (31) did not confirm this finding. It is likely that the relative amounts of cholesterol transported in chylomicrons and in intestinal VLDL are determined by the composition and amount of dietary fat, the amount of dietary cholesterol, and the rate of absorption.

Remnant metabolism in cholesterol-fed rabbits

Our data indicate that chylomicron-triglyceride hydrolysis, and thus remnant formation, is the same in cholesterol-fed and normal rabbits. In control animals, remnants are rapidly removed by the liver so that few remnants found in plasma have been reduced to particles smaller than large VLDL (VLDL-2). In hypercholesterolemic rabbits, hepatic remnant uptake is delayed, remnants circulate for much longer times, and a substantial fraction of the remnants is found among lipoproteins of $S_f < 20$.

A unique explanation for the accumulation of chylomicron remnants in plasma of cholesterol-fed rabbits is not possible at this time. It is known that rabbits absorb cholesterol efficiently, while hepatic enzymes that degrade cholesterol to bile acids appear to be less efficient in this species (32). At least two mechanisms could cause delayed hepatic uptake of remnants and result in elevated plasma remnant concentrations. It is possible that remnants accumulate due to saturation of the removal mechanism when the rate of dietary cholesterol input exceeds the normal, maximal, clearance rate. This saturation could occur at the level of conversion of hepatic cholesterol to bile acids, or at a hepatic receptor for remnants. Alternatively, an early consequence of cholesterol feeding could be impairment of the removal mechanism such that the efficiency of remnant removal is decreased.

When we examined the time course of chylomicron retinyl and cholesteryl ester radioactivity in two tissues, plasma and liver, we found that only 40–50% of the injected dose was present at early times (10 min), and that this value increased to 55–72% of the injected dose at times from 15 to 50 min. The early, rapid disappearance of labeled esters from plasma was the same in cholesterol-fed and control rabbits and coincided with the removal of chylo-

[3] Since submission of this paper, Fielding and Fielding (J. Lipid Res. **17:** 419–423. 1976) have demonstrated transfer of apolipoproteins, including the arginine-rich apoprotein, to rat lymph chylomicrons during short in vitro incubations with plasma.

micron triglyceride. We view this rapid decrease and subsequent increase in plasma retinyl and cholesteryl ester activity as compatible with the concept that chylomicrons are adsorbed to the vascular surface during lipolysis (33) and that, subsequently, remnants are released into the plasma compartment. This interpretation is supported by the additional observation that the *specific* radioactivity of plasma cholesteryl esters also reached a minimum at about 10 min and increased at subsequent time points (**Fig. 7**, cholesterol-fed rabbits). An increase in specific radioactivity is consistent with a model in which chylomicron remnant cholesteryl esters are recycled into plasma after temporary removal due to adsorption. In control rabbits, the specific activity–time curves showed a temporary upward deflection but seldom a net increase, probably due to more rapid hepatic clearance of released remnants.

Our finding that only 55–72% of chylomicron retinyl and cholesteryl ester radioactivity was in rabbit plasma and liver at times from 15–50 min is different from the results reported for normal rats in which nearly 90% of injected cholesteryl (6, 34) and retinyl esters (35) were recovered in these two tissues 20 min after chylomicron dosing. The lower recoveries in the rabbit are not due to entrapment of large chylomicrons in lung capillaries or to splenic phagocytosis. It is also unlikely that phagocytosis by Kupffer cells occurred, since only very small amounts of remnant radioactivity were found in liver soon after dosing. It appears that a substantial portion of remnant cholesteryl esters is removed by extrahepatic tissues in the rabbit. Consistent with extrahepatic remnant removal are our observations that hepatectomized control rabbits also remove a portion (up to 43%) of chylomicron retinyl and cholesteryl esters from plasma over a 1-hour time course.[4] Of interest, a lipoprotein lipase-related uptake of chylomicron cholesterol into rat mammary tissue has recently been observed by Zinder et al. (36).

Use of esterified retinol to trace degradation of intestinal chylomicrons

We consider the use of retinyl ester-labeled chylomicrons advantageous in distinguishing plasma lipoproteins of intestinal origin from those of hepatic origin. Retinyl ester, which is mainly retinyl palmitate (12), does not transfer from chylomicrons to other lipoproteins during incubation in vitro (below), and retinyl esters do not recirculate from liver in VLDL. Labeled free retinol recirculates from liver complexed to protein with a density greater than 1.21 and is therefore easily separated from plasma lipoproteins (11). Previously, large oral doses of vitamin A have been used by Beaumont, Ardaillou, and Lenegre (37) to study fat clearance in hyperlipidemic humans, while Hazzard and Bierman (38) have used labeled vitamin A to trace the disposition of dietary particles in type III hyperlipoproteinemic patients who appear to suffer from impaired remnant removal. Recently, Lewis et al. (39) have shown a decreased clearance of dietary vitamin A in a patient with autoimmune hyperlipidemia.

In animals, the use of an injected dose of doubly labeled chylomicrons has the potential to provide additional information about recirculation of labeled dietary cholesterol in endogenous lipoproteins. Recirculation should be apparent as an increase in the ratio of labeled cholesteryl esters to retinyl esters in plasma VLDL. In our studies, recirculation of labeled esterified cholesterol in VLDL was not observed within 2 hr of chylomicron injection. Where we have found small increases in the content of labeled esterified cholesterol relative to esterified retinol in whole plasma, these increases were limited to lipoproteins of $S_f < 20$. It seems most likely that this increase is due to exchange of a small portion of chylomicron cholesteryl esters with plasma lipoproteins of $S_f < 20$, as we have demonstrated in vitro. This transfer could be similar to the protein-catalyzed exchange of lipoprotein cholesteryl esters reported recently from this laboratory (40). In contrast to esterified cholesterol, recirculation of chylomicron retinyl esters from liver as free retinol was observed. Plasma collected at 90 min from control rabbits and cholesterol-fed rabbits contained, respectively, 3.8 and 2.2 times more free labeled retinol than that present in the injected dose.

Possible atherogenicity of remnants

The relative atherogenicity of different plasma lipoprotein classes is of considerable interest. The large proportions of VLDL in hypercholesterolemic rabbit plasma and of chylomicron remnants in VLDL, as well as the known susceptibility of this species to cholesterol-induced atheromatosis, suggest that the potential atherogenicity of chylomicron remnants deserves study. Indeed, it has been proposed recently that the process of remnant formation on the arterial surface could be instrumental in initiating aortic lesions (41).

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[4] A. C. Ross and D. B. Zilversmit. Unpublished results.

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