Effect of dietary cholesterol level on the composition of thoracic duct lymph lipoproteins isolated from nonhuman primates

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Abstract The effect of two different levels of dietary cholesterol (0.16 mg/Kcal and 0.79 mg/cal) on the composition of thoracic lymph duct lipoproteins was studied in two species of nonhuman primates, *Cercopithecus aethiops* (African green monkey) and *Macaca fascicularis* (cynomolgus monkey). Diet was infused intraduodenally at a constant rate to facilitate comparisons among animals. The higher level of dietary cholesterol resulted in an increase in the amount of cholesteryl ester in lymph chylomicrons and VLDL. Cholesteryl oleate was the predominant cholesteryl ester present in lymph d < 1.006 g/ml lipoproteins and it was the predominant cholesteryl ester formed from exogenous radiolabeled cholesterol. The percentage of saturated and monounsaturated cholesteryl esters in lymph chylomicrons and VLDL significantly increased with the higher dietary cholesterol level. The apoprotein distribution of chylomicrons and VLDL was qualitatively similar during infusions of both diets. The apoprotein B of intestinal chylomicrons and VLDL, termed apoprotein B2, was qualitatively similar during low and high cholesterol diet infusion and was significantly smaller than that of plasma LDL apoB, termed apoprotein B1, as indicated by its electrophoretic mobility in SDS-polyacrylamide gels. The major phospholipid present in lymph chylomicrons and VLDL was phosphatidylcholine and the phospholipid composition of the particles was not affected by diet. Lymph d > 1.006 g/ml lipoproteins were separated and the cholesterol mass distribution among lipoprotein fractions was found to be similar during both diet infusions. With an increase in the level of dietary cholesterol, the percentage esterification of cholesterol mass and of exogenous cholesterol radioactivity increased in LDL and HDL from lymph. Lymph LDL and HDL contained less free and esterified cholesterol when their composition was compared to that for these lipoproteins in plasma. We conclude that the primary effect of increased dietary cholesterol level was to increase the cholesteryl ester content of all lymph lipoproteins; cholesterol distribution among lymph lipoproteins was unaffected—**Klein, R. L., and L. L. Rudel.** Effect of dietary cholesterol level on the composition of thoracic duct lymph lipoproteins isolated from nonhuman primates. *J. Lipid Res.* 1983. **24:** 357–367.

Supplementary key words apolipoprotein • cholesteryl ester • chylomicron • exogenous cholesterol

We have reported in a separate publication (1) on studies carried out in nonhuman primates to determine the effects of increased dietary cholesterol level on cholesterol absorption and transport in lymph lipoproteins. We report here on the effects of dietary cholesterol level on lymph lipoprotein composition and distribution in the same population of nonhuman primates. In other laboratories, studies have been carried out in rats and dogs in which dietary cholesterol level has been shown to modify the composition of lymph lipoproteins (2–4). Zilversmit (2) fed dogs increasing levels of dietary cholesterol and found that the percentage of chylomicron lipid present as cholesteryl ester increased significantly. Free cholesterol content was also increased but not to the same extent as was cholesteryl ester. Fielding, Renston, and Fielding (3) found that chylomicron cholesteryl ester content was significantly increased in rats receiving intraduodenal lipid infusions enriched in cholesterol. Studies such as these show that increased dietary cholesterol level results in increased cholesteryl ester content of lymph lipoproteins; the cholesteryl ester increase was the main compositional difference and was of greater magnitude than the increase in the percentage of free cholesterol. In other studies in rats, Riley et al. (4) found a significantly increased percentage of cholesteryl ester in the lymph lipoproteins of the 1.006 < d < 1.063 g/ml density range which occurred together with a shift in cholesterol distribution that favored cholesterol transport in these lipoproteins. In sum, these studies have pointed out that the level of dietary cholesterol can alter the chemical composition and the cholesterol distribution among lymph lipoproteins. Such changes would be expected to result in al-

Abbreviations: ACAT, acyl CoA:cholesteryl acyl transferase; DTNB, 5,5′dithiobis (nitrobenzoic acid); EDTA, ethylenediamine tetraacetate; HDL, high density lipoproteins; HPLC, high performance liquid chromatography; LCAT, lecithin:cholesterol acyl transferase; LDL, low density lipoproteins; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins.

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terations among plasma lipoproteins *1*) by effects on liver cholesterol metabolism since chylomicron cholesteryl esters are rapidly cleared from plasma by the liver (5–7) and *2*) by effects on intravascular metabolism since chylomicron surface is the precursor material for HDL (8, 9). In species such as the rabbit, it is possible that dietary cholesterol-induced compositional changes in lymph lipoproteins can retard their clearance rate from plasma (10).

The present studies were carried out to define the changes in lymph lipoproteins associated with the diet-induced modifications in plasma lipoproteins in nonhuman primates. These animals have a characteristic cholesteryl ester enrichment of their plasma LDL that occurs in response to cholesterol feeding (11, 12). The extent of this change was found to be highly correlated to the degree of coronary artery atherosclerosis (13, 14). The compositional analyses of lymph lipoproteins of the present study suggest that the cholesteryl ester-enriched plasma LDL of nonhuman primates does not arise directly from intravascular metabolism of lymph chylomicrons and VLDL, but rather, LDL enlargement may result indirectly presumably as a consequence of hepatic metabolism of cholesteryl ester enriched chylomicrons and VLDL.

METHODS AND MATERIALS

Two species of nonhuman primates were used in these studies, namely cynomolgus macaques (*Macaca fascicularis*) and African green monkeys (*Cercopithecus aethiops*). The thoracic lymph duct was exposed and catheterized resulting in a continuous closed-loop of lymph flow and, in some animals, an intraduodenal catheter was also surgically implanted for subsequent infusion of diets (1). The animals were permitted to recover from surgery in individual animal cages for 10–14 days before an experiment was begun. In most of the experiments, each animal was infused with two fat-rich diets that differed only in the cholesterol level of the diet (1). In addition, several animals were fed in sequence solid diets with low and then high cholesterol levels (the 75-8B diets of ref. 15).

Radiolabeled cholesterol was purchased from one of three companies, Research Products International, Elk Grove, IL; New England Nuclear, Boston, MA; or Amersham, Arlington Heights, IL. Each lot was purified and then added to experimental diets as described previously (1). Aliquots of the organic solvent extracts of lymph, of lipoprotein solutions, and of free and esterified cholesterol used in cholesterol mass analyses were taken for determination of radioactivity in a Beckman LS-7000 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA) as described previously (1).

Thoracic duct lymph was collected and lymph d < 1.006 g/ml lipoproteins were subsequently isolated as described previously (1). In some experiments, the $1.006 < d < 1.225$ g/ml lipoprotein fraction was isolated from lymph as follows. Approximately 200 ml of lymph collected during the last 12 hr of each infusion were overlaid with saline and spun in the SW-27 rotor at 27,000 RPM for 24 hr at 15°C. The floating d < 1.006 g/ml lipoprotein fraction of lymph was removed from the centrifuge tube, and lymph containing the d > 1.006 g/ml lipoprotein fraction was then concentrated at 4°C (Amicon, Model 52, UM-20 membrane; Lexington, MA) to approximately 20–40 ml. The $1.006 < d < 1.225$ g/ml lipoproteins were then isolated by ultracentrifugation and were separated using agarose column chromatography (Bio-Gel® A 15 m, 200–400 mesh, Bio-Rad, Rockville Center, NY) as described for plasma lipoproteins by Rudel et al. (16).

Blood samples were routinely obtained from each animal before and after each experiment. The samples were collected in 0.1% EDTA, 0.02% azide, 0.04% DTNB, pH 7.4 (final concentration). Plasma was obtained after low speed centrifugation of the blood samples and lipoproteins were then isolated as described previously (16). Lipoprotein fractions were extracted with chloroform–methanol 2:1, lipid classes were separated by thin-layer chromatography, and analyses of individual classes were performed as previously described (13). Apolipoproteins were extracted with ethanol–ether 3:1, separated by polyacrylamide gel electrophoresis in 0.1% SDS, and stained with Coomassie blue as previously described (13).

Individual cholesteryl esters were separated and quantitated using reverse phase high performance liquid chromatography (HPLC). Cholesteryl esters of all lymph lipoprotein samples for HPLC analysis were first isolated by preparative thin-layer chromatography with silica gel H because the amount of triglyceride found in lymph lipoprotein total lipid extracts interfered with the subsequent HPLC cholesteryl ester separation. The HPLC analyses were performed using the chromatography conditions described by Carroll and Rudel (17). Radioactivity in the cholesterol moiety of the cholesteryl ester was monitored using a radioactive flow detector (Flo-One®, Radiomatic Instruments and Chemical Co., Inc., Addison, IL). The HPLC column eluate was automatically and continuously mixed with a liquid scintillation cocktail (Budget-Solve®, Research Products International Corp., Elk Grove Village, IL) to monitor cholesteryl ester radioactivity.

The separation of lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, and

phosphatidylethanolamine was performed with modifications of the procedure of Skipski (18). The sample was spotted on silica gel thin-layer chromatography plates (Silica Gel 60, F-254, precoated; 0.25 mm-thick; Brinkmann, Chicago, IL). The plates were first developed in hexane–acetone 3:1 (v/v) and then were developed in chloroform–methanol–glacial acetic acid–water 65:45:12:6 (v/v). Phospholipids were visualized using iodine vapor. Areas of silica gel containing phospholipid were scraped into tubes and phospholipid was eluted using chloroform–methanol–7 N NH_4OH 65:35:5 (v/v). Appropriate aliquots from each tube were then dried and phospholipid phosphorus was assayed (19). Phospholipid phosphorus recovery from the plate averaged 85%.

Data in the study were analyzed using the paired *t*-test essentially as described by Snedecor and Cochran (20).

RESULTS

Percentage composition of lymph lipoproteins

The influence of the level of dietary cholesterol on the percentage composition of lymph chylomicrons and VLDL is shown by the data in **Table 1.** Data from African green and cynomolgus monkeys were averaged together because no species differences were found. The percentage of cholesteryl ester in both chylomicrons and VLDL increased with the higher level of dietary cholesterol. The percentage of free cholesterol in both lipoproteins was also increased during the high cholesterol diet infusion. The percentages of phospholipid, triglyceride, and protein were similar regardless of the level of dietary cholesterol. The percentage value of any one constituent depends on the relative amounts of the other constituents and therefore cannot be simply and directly compared by a *t*-test or other statistical analysis requiring a normal distribution. Ratios of constituents, instead of percentages, were therefore used for statistical analysis by paired *t*-tests of chemical composition differences since the values for individual ratios, determined from the measured weights, are independent of each other and were normally distributed. With the high level of dietary cholesterol, the total cholesterol content of both chylomicrons and VLDL was increased significantly as indicated by the decrease in TG/TC. The increase in the total cholesterol content of the chylomicrons was due to a statistically significant increase in both the free cholesterol and esterified cholesterol components of the particles during the high cholesterol diet infusion as indicated by the changes in TG/FC and TG/EC. In the VLDL, the free cholesterol content of the particle increased slightly as indicated by the change

TABLE 1. *Influence of the level of dietary cholesterol on the composition of lymph chylomicrons and VLDL*

	% by Weight					Ratios of Constituents (w/w)						
	FC	CE	PL	TG	Pro	$\frac{TG}{TC}$	$\frac{EC}{TC}$	$\frac{TG}{FC}$	$\frac{TG}{EC}$	$\frac{TG}{PL}$	$\frac{TG}{Pro}$	$\frac{PL}{Pro}$	$\frac{PL}{FC}$
Chylomicrons													
Low cholesterol meal	0.74[a] ± 0.05	1.25 ± 0.09	7.81 ± 0.22	88.72 ± 0.31	1.48 ± 0.05	63.76 ± 4.34	0.48 ± 0.02	127.20 ± 10.41	127.97 ± 8.86	11.44 ± 0.39	61.01 ± 2.32	5.25 ± 0.26	10.99 ± 0.82
High cholesterol meal	0.87 ± 0.03	1.82 ± 0.09	8.27 ± 0.51	87.56 ± 0.49	1.47 ± 0.07	46.66[c] ± 1.40	0.55[c] ± 0.01	104.27[b] ± 4.18	84.60[d] ± 3.73	11.16[c] ± 0.54	61.06[c] ± 9.56	5.45[c] ± 0.71	9.45[c] ± 0.51
VLDL													
Low cholesterol meal	1.73 ± 0.08	3.01 ± 0.18	17.15 ± 0.42	73.39 ± 0.49	4.71 ± 0.13	21.25 ± 0.98	0.50 ± 0.02	43.75 ± 2.32	42.91 ± 2.45	4.28 ± 0.13	15.74 ± 0.56	3.63 ± 0.40	10.11 ± 0.59
High cholesterol meal	1.92 ± 0.01	4.46 ± 0.05	16.93 ± 0.47	72.01 ± 0.79	4.71 ± 0.18	16.90[b] ± 1.28	0.57[b] ± 0.02	39.10[c] ± 1.86	30.71[c] ± 3.25	4.32[c] ± 0.18	15.66[c] ± 0.59	3.55[c] ± 0.40	9.04[c] ± 0.48

[a] Mean ± SEM for ten animals; six observations averaged for each animal.
[b] $P < 0.05$; low vs. high.
[c] $P < 0.01$; low vs. high.
[d] $P < 0.001$; low vs. high.
[e] Not significant; low vs. high.
Abbreviations: TC, total cholesterol; FC, free cholesterol; EC, esterified cholesterol; CE, cholesteryl ester = EC × 1.7; PL, phospholipid; TG, triglyceride; Pro, protein.

TABLE 2. Phospholipid composition of lymph d < 1.006 g/ml lipoproteins

Animal	% by Weight				
	LPC[a]	SM	PC	PS	PE
66	2.9	2.0	76.4	2.3	16.4
144	2.1	2.0	79.2	1.7	15.0
439	3.0	1.2	78.7	1.8	15.3
Mean ± SEM	2.7 ± 0.3[b]	1.7 ± 0.3	78.1 ± 0.9	1.9 ± 0.2	15.6 ± 0.4

[a] Abbreviations are: LPC = lysophosphatidylcholine, SM = sphingomyelin, PC = phosphatidylcholine, PS = phosphatidylserine + phosphatidylinositol, PE = phosphatidylethanolamine.
[b] Mean ± SEM for three animals; four to eight observations for each animal.

in TG/FC ratio, but this change was not statistically significant. However, the esterified cholesterol content of the VLDL was significantly increased with the high cholesterol diet infusion as evidenced by the significantly decreased TG/EC ratio. The proportion of the cholesterol in the particles that was esterified also changed with the percentage of esterified cholesterol (EC/TC ratio) increasing from 48% to 55% and from 50% to 57% in the chylomicrons and VLDL, respectively, during the high cholesterol diet infusion. These increases were statistically significant for both lipoproteins.

The chemical composition was also determined for chylomicrons and VLDL isolated from sequential samples obtained during three self-feeding studies. The percentage composition of both the chylomicrons and VLDL changed with the increase in the level of dietary cholesterol in a manner similar to that observed during the constant infusion studies. Statistical analysis using the TG/TC ratio of the lipoproteins indicated that, with the high level of dietary cholesterol, the total cholesterol of both chylomicrons and VLDL had significantly increased ($P < 0.01$), i.e., the TG/TC ratio decreased. This increase in the total cholesterol of the particles was primarily due to a significant increase ($P < 0.01$) in esterified cholesterol as evidenced by the lower TG/EC ratio; the TG/FC ratio was also decreased, but statistical significance at the 5% level was not reached.

The phospholipid composition of the chylomicrons and VLDL from sequential samples obtained during both meals was determined using thin-layer chromatography. The phospholipid composition of the lipoproteins was not affected by diet, lipoprotein size, or time after a meal; therefore all data were combined in **Table 2.**

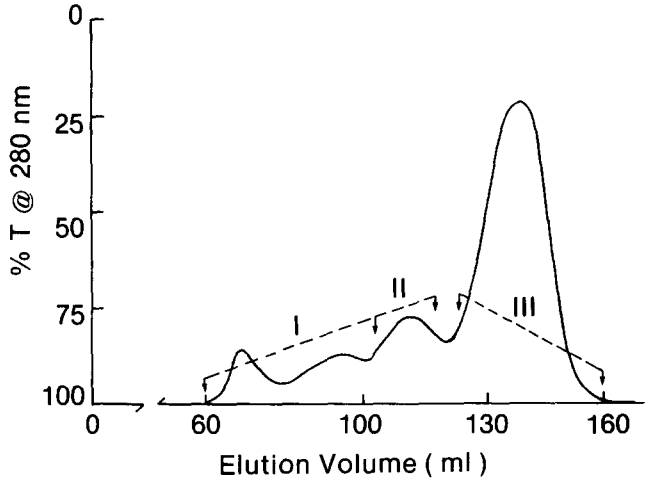

Fig. 1. Agarose column chromatography elution profile of the 1.006 < d < 1.225 g/ml lipoprotein fraction of lymph from a single animal. Samples were prepared as detailed in Methods section. The material in the regions indicated by the Roman numerals was then pooled for further analysis. The profile is for lymph obtained during the 24–36-hr period of a high cholesterol diet infusion.

Cholesterol transport in d > 1.006 g/ml lipoproteins

A representative agarose column chromatography elution profile showing the size distribution of the lymph d > 1.006 g/ml lipoproteins is shown in **Fig. 1.** The mass of material eluting in the first peak (near the void volume) was very low, therefore the first two peaks were pooled as region I. The elution volumes for regions II and III corresponded to those of plasma LDL and HDL, respectively. The cholesterol mass distribution for the three regions was similar during both diet infusions and averaged 14.5%, 40.9%, and 44.6% for regions I, II, and III, respectively, in five animals. Exogenous cholesterol radioactivity was distributed similarly during both diet infusions and averaged 12.6%, 27.1%, and 60.3% in regions I, II, and III, respectively in five animals. The percentage of exogenous cholesterol radioactivity in the esterified form significantly increased ($P < 0.05$) from 50.0% to 62.4% in region II lipoproteins and from 35.4% to 49.8% in region III lipoproteins during the high cholesterol diet infusion.

As indicated by the data in **Table 3,** during the high cholesterol diet infusion, a greater proportion of the cholesterol in every component of region II and III

TABLE 3. Influence of the level of dietary cholesterol on the percentage of cholesterol derived from exogenous origin in lymph lipoproteins of agarose column regions II and III

	Exogenous Cholesterol[a] (%)		
	Low Cholesterol Diet	High Cholesterol Diet	Significance
Region II lipoproteins			
FC	7.0 ± 1.7[b]	22.7 ± 4.5	<0.05
EC	1.1 ± 0.7	4.3 ± 0.8	N.S.
Significance, FC vs. EC	$P = 0.05$	<0.001	
Region III lipoproteins			
FC	8.6 ± 1.2	23.9 ± 5.2	<0.05
EC	2.2 ± 0.8	12.9 ± 2.8	<0.05
Significance, FC vs. EC	<0.05	<0.001	

[a] These values were calculated as described in ref. 1. The lymph samples were obtained when the cholesterol specific activity in lymph had become constant relative to that of the diet infused, so that the percentage of exogenously derived cholesterol can be calculated as the lipoprotein FCSA or ECSA ÷ dietary cholesterol SA (×100).
[b] Mean ± SEM for four to six animals, one observation per animal.
Abbreviations as given in Table 1.

lipoproteins was of exogenous origin than was the case during the low cholesterol diet infusion. Regardless of the level of dietary cholesterol, a greater proportion of free cholesterol was of exogenous origin in both lipoprotein fractions than was the case for esterified cholesterol. The percentage of region III free cholesterol derived from exogenous origin was significantly greater ($P < 0.05$) than the percentage of that of region II regardless of the level of dietary cholesterol. This was true also for the percentage of region III esterified cholesterol ($P < 0.05$).

The chemical composition data of lymph region II and III lipoproteins are found in **Table 4.** Relative to plasma LDL, lymph region II lipoproteins were protein- and phospholipid-rich particles deficient in cholesteryl esters and free cholesterol. There was a greater weight percentage of triglyceride in the region II lipoproteins compared to plasma LDL. The ratios of chemical constituents suggest that there was an increase in both free and esterified cholesterol with more of the cholesterol present in the esterified form during the high cholesterol diet infusion. Statistical analyses were not performed in view of the limited number of samples.

Region III lymph lipoproteins were also lowered in the amount of free and esterified cholesterol as compared to plasma HDL and were relatively higher in the amount of phospholipid. The percentage of triglyceride in region III lipoproteins was greater than that in plasma HDL. An increased fraction of total cholesterol was present in the esterified form during the high cholesterol diet infusion in region III lipoproteins.

Apolipoprotein pattern of appearance

SDS-PAGE was performed on apolipoproteins of chylomicrons and VLDL from sequential lymph samples obtained during both meals. The apoprotein composition of the chylomicrons and VLDL isolated during the self-feeding studies was similar to that seen in samples obtained during constant infusion studies. No dietary cholesterol-induced differences in the apoprotein patterns of lymph chylomicrons and VLDL were apparent. Representative data from a single animal are shown in **Fig. 2.** The apoprotein distributions of the chylomicrons were similar for each timed lymph collection; the same

TABLE 4. Influence of the level of dietary cholesterol on the composition of lymph region II and III lipoproteins

	% by Weight					Ratios of Constituents (w/w)					
	FC	CE	PL	TG	Pro	TC/ Pro	EC/ TC	FC/ Pro	EC/ Pro	PL/ Pro	TG/ Pro
Region II											
Low cholesterol[a] meal	6.9 ± 4.7	23.9 ± 9.2	28.9 ± 8.4	15.7 ± 1.7	24.8 ± 2.3	0.85 ± 0.06	0.64 ± 0.30	0.20 ± 0.16	0.55 ± 0.24	1.21 ± 0.64	0.63 ± 0.01
High cholesterol meal	5.1 ± 1.6	26.4 ± 6.3	28.7 ± 3.3	16.5 ± 3.6	23.4 ± 2.1	0.97 ± 0.10	0.80 ± 0.14	0.32 ± 0.26	0.74 ± 0.02	1.25 ± 0.36	0.72 ± 0.31
Plasma LDL[b]	8.6 ± 0.1	46.4 ± 0.1	24.2 ± 0.1	3.5 ± 0.1	17.4 ± 0.1	2.07 ± 0.02	0.76 ± 0.01	0.50 ± 0.02	1.57 ± 0.04	1.39 ± 0.01	0.20 ± 0.05
Region III											
Low cholesterol[a] meal	2.2 ± 1.0	5.2 ± 0.4	35.7 ± 5.0	6.7 ± 1.6	50.2 ± 7.2	0.11 ± 0.04	0.50 ± 0.19	0.04 ± 0.02	0.06 ± 0.01	0.74 ± 0.29	0.14 ± 0.07
High cholesterol meal	2.0 ± 1.0	4.9 ± 0.1	34.5 ± 0.2	8.2 ± 2.8	50.6 ± 3.1	0.10 ± 0.02	0.65 ± 0.06	0.05 ± 0.03	0.06 ± 0.01	0.68 ± 0.06	0.17 ± 0.09
Plasma HDL[b]	3.2 ± 0.1	18.8 ± 0.2	28.4 ± 0.1	1.3 ± 0.3	48.3 ± 0.1	0.30 ± 0.01	0.78 ± 0.02	0.07 ± 0.03	0.23 ± 0.02	0.59 ± 0.02	0.03 ± 0.03

[a] Mean ± S.D. of single determinations on samples from two animals.
[b] Mean ± S.D. of single determinations on plasma lipoprotein samples obtained from the same two animals before lymph duct cannulation.
Abbreviations as given in Table 1.

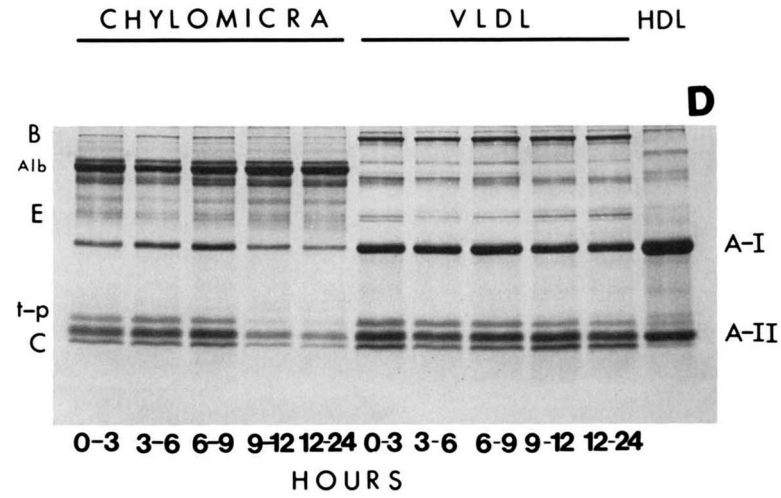

Fig. 2. The 12.5% polyacrylamide-SDS gel electrophoresis patterns of apoproteins of chylomicrons and VLDL sequentially isolated during the course of a low cholesterol level meal in animal #66. The apoprotein pattern of HDL isolated from plasma is shown for reference. Approximately 40 μg of protein was applied in each well. The apoprotein components identified were apoproteins B, E, A-I, A-II, and C, as judged by their comigration with each of these apoproteins in purified form. The tentatively identified threonine-poor apoprotein is indicated by 't-p'. The position of albumin migration is indicated by Alb.

was true for VLDL. The relative amount of protein in the albumin region was quite different between the chylomicrons and VLDL; the other bands in this region may be apoproteins A-IV and A-V. Further centrifugal washing of chylomicrons was not carried out because this resulted in detectable losses for each of the apoproteins to the infranate, not just removal of albumin. The intensely staining band migrating slightly slower than the A-II and C apoproteins has been tentatively identified as the threonine-poor apoprotein as described by Parks and Rudel (21).

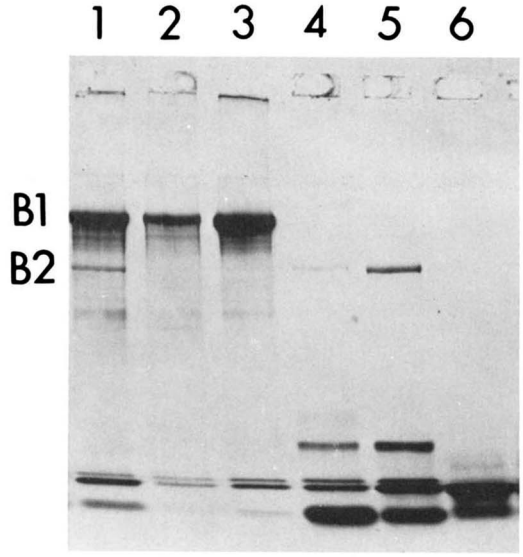

Fig. 3. The 5% polyacrylamide-SDS gel electrophoresis patterns of apoproteins isolated from 1) lymph region II lipoproteins, 2) plasma LDL isolated after 4 days of thoracic duct lymph drainage, 3) normal plasma LDL isolated from the same animal before lymph drainage, 4) lymph chylomicra, 5) lymph VLDL, and 6) normal plasma HDL. All fractions are from the same animal. Approximately 40 μg of protein was applied in each well. The two forms of apoprotein B are indicated as B1 and B2; both were cross-reactive with antisera to plasma LDL (apoB1). The remainder of the apoproteins migrated to the bottom of the gel and were not identified.

The apoprotein B of lymph and plasma lipoproteins is better visualized by using PAGE with a 5% separating gel as shown for a representative animal in **Fig. 3.** The lymph chylomicrons and VLDL have a single predominant apoprotein B band (apoB2) which migrated through the spacer gel and about 2 cm into the separating gel; dietary cholesterol level did not alter this pattern. The apoprotein B of normal plasma LDL (apoB1) also penetrated the separating gel but with a mobility less than that of apoB2. These two forms of apoprotein B coincide with the two forms of apoB that have been described for rats and humans (22, 23), and both react with antisera to monkey plasma LDL during double immunodiffusion. The apoprotein B of LDL isolated from a plasma sample taken after 4 days of lymph drainage co-migrated with the slower migrating apoB1 band of normal plasma LDL; only a very faint band in the apoB2 region was found in either of these plasma LDL samples. The region II lymph lipoproteins contained predominantly the slower migrating apoB1 component but in contrast to the data for plasma LDL, the apoB2 component was also clearly detectable in lymph LDL.

The apoprotein composition of plasma HDL and lymph region III lipoproteins is shown in the electrophoretogram in **Fig. 4.** The predominant apoproteins

of these lipoproteins were apoproteins A-I and A-II; some apoprotein C is present but no apoprotein B was found in these lipoproteins. The level of dietary cholesterol did not appear to consistently modify the apoprotein composition of the region III lymph lipoproteins, although for the animal of Fig. 4 there was relatively less apoA-II and threonine-poor apoprotein in the sample taken from lymph during the low cholesterol meal (gel 3). The region III lymph lipoprotein apoprotein distribution was similar to that of plasma HDL. The apoproteins of HDL isolated from plasma after 4 days of lymph drainage were similar to those of normal plasma HDL except for the increased amount of threonine-poor apoprotein. The threonine-poor apoprotein was typically present in the lipoproteins isolated from animals during periods of chair restraint.[2]

Lymph lipoprotein cholesteryl esters

The distribution of lymph lipoprotein cholesteryl esters was determined using high performance liquid chromatography (HPLC). The analysis of cholesteryl esters using HPLC does not require hydrolysis of the ester linkage. Therefore, the distribution of radiolabeled exogenous cholesterol in cholesteryl esters could also be determined using HPLC. Cholesterol radioactivity was monitored using an on-line liquid scintillation detector. A representative chromatogram showing the separation of chylomicron cholesteryl esters and the distribution of exogenous cholesterol radioactivity in the intact cholesteryl esters is shown in **Fig. 5.** A peak with a retention time similar to that of free cholesterol was seen with reasonable regularity in cholesteryl ester samples from lymph lipoproteins. The peak did not contain radioactivity and was probably not free cholesterol but may be a retinol derivative, or possibly another fat-soluble vitamin.

The cholesteryl ester composition of d < 1.006 g/ml lymph lipoproteins isolated during both infusions is found in **Table 5.** The predominant cholesteryl ester in both chylomicrons and VLDL during both infusions was cholesteryl oleate. The percentage of cholesteryl palmitate and cholesteryl oleate in both lipoproteins appeared to increase during the high cholesterol diet infusion. This observation was confirmed by the statistically significant increase in the ratio of the saturated and monounsaturated cholesteryl esters relative to those cholesteryl esters with two or more unsaturated double bonds in their fatty acid moiety [(Δ0 + Δ1)/Δ2 +]. The ratio of cholesteryl oleate to cholesteryl linoleate was significantly higher in animals receiving the high cholesterol diet. The relative proportions of the other saturated and monounsaturated cholesteryl esters remained similar during both infusions, suggesting there was a proportionate increase in appearance rate in each

[2] Parks, J. S., and L. L. Rudel. Manuscript in preparation.

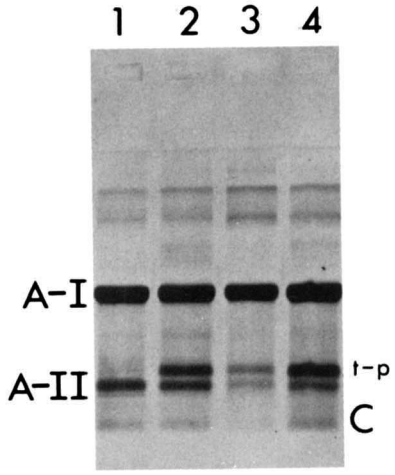

Fig. 4. The 12.5% polyacrylamide-SDS gel electrophoresis patterns of apoproteins isolated from: 1) normal plasma HDL isolated before lymph drainage, 2) HDL from a plasma sample collected after 4 days of lymph drainage, 3) region III lymph lipoproteins isolated during low cholesterol level meal, 4) region III lymph lipoproteins isolated during the high cholesterol meal. Each lipoprotein fraction was isolated from the same animal. The apoproteins identified were apoproteins A-I, A-II, C, and the threonine-poor apoprotein as given in Fig. 2. Approximately 40 μg protein was applied in each well.

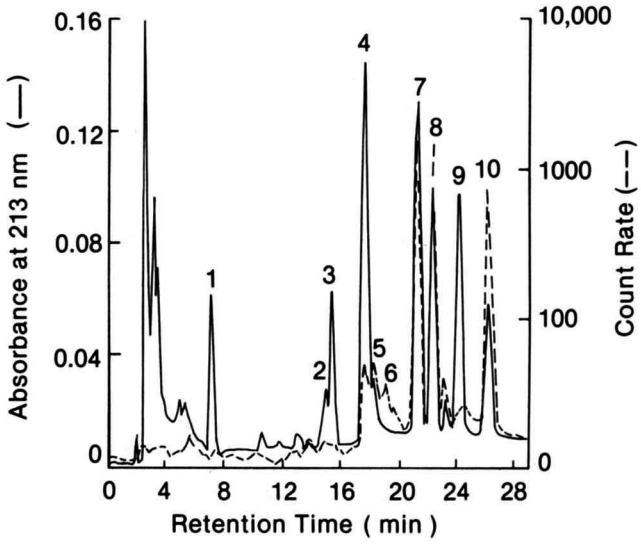

Fig. 5. A comparison of the radioactivity versus mass distribution of lymph chylomicron cholesteryl esters after separation by high performance liquid chromatography (HPLC). Lymph chylomicrons were isolated during the infusion of a low cholesterol level diet containing [^{3}H]cholesterol. Approximately 250 μg of esterified cholesterol was applied to the column. Cholesteryl ester mass (——) was monitored spectrophotometrically at 213 nm. Cholesteryl ester radioactivity (– – –) was determined by in-line scintillation counter. Peak identification: 1) free cholesterol, 2) cholesteryl linolenate, 3) cholesteryl arachindonate, 4) cholesteryl linoleate, 5) cholesteryl palmitoleate, 6) cholesteryl myristate, 7) cholesteryl oleate, 8) cholesteryl palmitate, 9) cholesteryl heptadecanoate, and 10) cholesteryl stearate.

TABLE 5. Influence of the level of dietary cholesterol on cholesteryl ester composition of lymph lipoproteins

	Cholesteryl Ester (%)								Ratios of Constituents (w/w)				
	CMy[c]	CPa	CPo	CSt	COl	CLn	CLl	CAr	$\frac{\Delta 0 + \Delta 1}{\Delta 2+}$	$\frac{COl}{CLn}$	$\frac{COl}{CSt}$	$\frac{CPa}{CSt}$	$\frac{COl}{CPa}$
Chylomicrons													
Low cholesterol meal	1.50[a]	19.10	4.97	10.37	42.86	23.70	0.80	1.10	2.93	1.71	4.31	1.92	2.32
	± 0.40	± 0.47	± 0.25	± 0.61	± 0.97	± 1.12	± 0.24	± 0.17	± 0.16	± 0.12	± 0.31	± 0.10	± 0.11
High cholesterol meal	1.19	22.68	3.13	11.05	44.99	17.99	0.66	0.54	4.42[b]	2.40[b]	4.14	2.11	2.03
	± 0.18	± 0.62	± 0.53	± 1.03	± 0.81	± 0.98	± 0.08	± 0.06	± 0.36	± 0.14	± 0.53	± 0.31	± 0.11
VLDL													
Low cholesterol meal	0.86	20.97	3.16	12.39	38.86	24.49	0.69	1.46	2.84	1.62	3.61	1.91	1.93
	± 0.15	± 0.75	± 0.46	± 1.66	± 0.90	± 1.59	± 0.04	± 0.10	± 0.23	± 0.11	± 0.53	± 0.20	± 0.11
High cholesterol meal	1.39	23.02	3.60	11.53	43.02	18.24	0.64	0.82	4.35[b]	2.40[b]	3.74	2.00	1.82
	± 0.18	± 0.78	± 0.36	± 1.06	± 1.07	± 1.31	± 0.04	± 0.11	± 0.39	± 0.14	± 0.51	± 0.21	± 0.11

[a] Mean ± SEM for six animals; two to nine observations for each animal.
[b] $P < 0.01$; low vs. high.
[c] Abbreviations: CMy, cholesteryl myristate; CPa, cholesteryl palmitate; CPo, cholesteryl palmitoleate; CSt, cholesteryl stearate; COl, cholesteryl oleate; CLn, cholesteryl linoleate; CLl, cholesteryl linolenate; CAr, cholesteryl archidonate. Δ0, Δ1, and Δ2+, cholesteryl esters with 0, 1, or ≥2 double bonds in the fatty acid, respectively.

of these cholesteryl esters during the high cholesterol diet infusion.

The specific activity of individual cholesteryl esters of lymph lipoproteins is shown in **Table 6.** The specific activity of an individual ester was essentially equivalent when the value for chylomicrons is compared to that for VLDL although in some cases the VLDL CESA was significantly lower. In only one case, cholesteryl stearate, was the VLDL CESA significantly higher than that for chylomicrons. The specific activity for each of the individual cholesteryl esters was significantly higher when the high cholesterol diet was fed ($P < 0.001$). The cholesteryl ester with the lowest specific activity was, without exception, cholesteryl linoleate. A paired *t*-test was used to compare the specific activity of cholesteryl linoleate to that of cholesteryl palmitate, cholesteryl stearate, and cholesteryl oleate. In each case, the lower specific activity of cholesteryl linoleate was statistically significant. The specific activity of cholesteryl arachidonate was also compared in this way and the difference was also found to be statistically significant. The cholesteryl linoleate specific activity was lower than that of cholesteryl arachidonate in both chylomicrons and VLDL but the difference was statistically significant only while the animals were fed the high cholesterol diet.

DISCUSSION

In the companion studies to the work presented here, we showed that increased dietary cholesterol levels resulted in an increase in lymphatic transport of exogenous cholesterol as cholesteryl ester (1). The data suggested that cholesterol esterification, presumably by intestinal ACAT, was likely to play an important role in regulation of the amount of cholesterol absorption. The data in this paper reinforce this possibility. The high cholesterol diet infusion resulted in the appearance of chylomicrons and VLDL that were cholesteryl ester-enriched (Table 1) while the pattern of exogenous cholesterol distribution among cholesteryl esters (Table 6) suggested that these esters had been synthesized by ACAT (24). The free cholesterol content of the lymph lipoproteins was also increased by the higher dietary cholesterol level but not to the extent that cholesteryl ester content was increased. These findings clearly indicate that increased cholesterol esterification accompanies increased dietary cholesterol absorption in primates as has been shown to be the case in lower animal species (25, 26). Although the lipid composition of lymph lipoproteins was significantly altered by a high level of dietary cholesterol, the apoprotein composition of lymph lipoproteins appeared unaltered. The apoprotein composition of chylomicrons and VLDL was qualitatively similar regardless of the level of dietary cholesterol. This was true for constant infusion studies as well as for the self-feeding studies.

In nonhuman primates fed dietary cholesterol with saturated fat, hypercholesterolemia is associated with the enrichment of plasma LDL with saturated and monounsaturated cholesteryl esters (13, 27). However, based on the data of these experiments, two lines of evidence suggest that intestinal chylomicrons and VLDL are probably not direct precursors of cholesteryl ester-enriched plasma LDL. Firstly, the plasma LDL cholesteryl ester composition (17) was considerably different from that found for the lymph lipoproteins (Table 5). There were proportionately more cholesteryl palmitate, cholesteryl stearate, and cholesteryl oleate and less cholesteryl linoleate in lymph lipoproteins than was found

TABLE 6. Lymph lipoprotein cholesteryl ester specific activity during infusion of low and high cholesterol level diets[a]

	Cholesteryl Ester Specific Activity (cpm/μg)								Significance[d]							
									CLn versus				CAr versus			
	CMy[c]	CPa	CPo	CSt	COl	CLn	CLl	CAr	CPa	CSt	COl	CPa	CSt	COl	CLn
Chylomicrons															
Low cholesterol meal	343[b] ± 134	330 ± 80	84 ± 11	242 ± 67	220 ± 46	38 ± 5	150 ± 47	89 ± 30	<0.001	<0.001	<0.001	<0.01	<0.05	<0.05	NS
High cholesterol meal	1162 ± 329	760 ± 94	604 ± 236	614 ± 114	585 ± 81	115 ± 18	322 ± 66	329 ± 57	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001
VLDL															
Low cholesterol meal	323 ± 139	260 ± 46	115 ± 19	251 ± 66	186 ± 40	36 ± 9	148 ± 19	43 ± 10	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS
High cholesterol meal	682 ± 128	755 ± 138	331 ± 91	631 ± 146	559 ± 109	109 ± 32	423 ± 75	205 ± 61	<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.01
Significance—chylomicrons vs. VLDL															
Low cholesterol meal	NS	<0.01	NS	NS	<0.01	NS	NS	NS							
High cholesterol meal	<0.05	NS	<0.01	<0.01	<0.05	NS	NS	NS							

[a] Cholesteryl ester specific activity during low cholesterol diet infusion was normalized to high cholesterol diet specific activity.
[b] Mean ± SEM for six animals; two to nine observations on each animal.
[c] Abbreviations are the same as for Table 5.
[d] Significance was determined by paired *t*-test, except where variance was not normally distributed, in which case the nonparametric sign rank test was substituted.

in plasma LDL. In addition, there was approximately 2–4 times more cholesteryl ester per particle in chylomicrons than in LDL, as evidenced by estimating the particle size and molecular weight of the chylomicrons from the percentages of the surface constituents (28). Extensive modification of lymph lipoprotein cholesteryl ester composition would be required if intestinal lipoproteins were to serve as a precursor pool to plasma LDL during cholesterol feeding. The second line of evidence is based on the observed apoprotein B heterogeneity. The electrophoretic mobility in SDS-polyacrylamide gels of apoprotein B from intestinal chylomicrons and VLDL (apoB2) was significantly different than that from plasma LDL (apoB1) (Fig. 3). Since it is currently believed that the apoprotein B molecule of one lipoprotein particle is not exchangeable to another particle, the intestinal chylomicron and VLDL particle per se probably is not directly converted to a plasma LDL particle (29).

The cholesteryl ester specific activity was similar for each cholesteryl ester present as a major constituent except cholesteryl linoleate (Table 6). Approximately 21% of the cholesteryl ester mass of chylomicrons and VLDL was composed of cholesteryl linoleate, yet less than 5% of the absorbed dietary radioactive cholesterol in lymph lipoprotein cholesteryl ester was found in this cholesteryl ester. The pattern of labeling of lymph lipoprotein cholesteryl esters was consistent with the specificity of intestinal ACAT (24), i.e., greater than 90% of the ester cholesterol radioactivity present was in cholesteryl palmitate (32%), oleate (45%), and stearate (13%), each having a similar specific activity. It seems reasonable to assume that the free cholesterol available to the esterifying enzyme (ACAT) was equally available as a substrate for synthesis of all cholesteryl esters, i.e., the availability of fatty acid substrates would not affect cholesteryl ester specific activity. Thus the primary determining factor of individual cholesteryl ester specific activity would be the free cholesterol specific activity of the ACAT substrate pool of free cholesterol. In this situation, the finding of lower cholesteryl linoleate specific activity suggests that a significant proportion of cholesteryl linoleate may have been contributed to the lipoprotein after it was secreted from the mucosal cell. LCAT activity has been identified in lymph of rats (30), and we found cholesteryl ester transfer activity in monkey lymph.[3] To rule out in vitro modifications after lymph collection, DTNB was added to all lymph samples and several lymph samples were collected at 4°C. The cholesteryl ester percentage distribution of chylomicrons collected at 4°C was similar to that for lipopro-

[3] Klein, R. L., L. L. Rudel, and M. S. Thomas. Unpublished observation.

teins collected at room temperature suggesting that there was minimal modification of lipoprotein cholesteryl ester composition in the collection vessel. It appeared that endogenous cholesteryl linoleate was transferred into chylomicrons and VLDL in the lymphatics. This would mean that lipoproteins isolated from thoracic duct lymph should not be considered nascent lipoproteins; in fact, they may have undergone considerable metabolic alteration while still in lymph.

The high cholesterol diet infusion resulted in a significant cholesterol transport rate increase of 1.78 mg/hr in the d < 1.006 g/ml lymph lipoproteins (1) but the increase in d > 1.006 lipoproteins was only 0.11 mg/hr and was not statistically significant. Although the d > 1.006 g/ml lymph lipoproteins were cholesteryl ester-enriched during the high cholesterol diet infusion (Table 4), the proportion of lymph cholesterol present in these lipoproteins was similar during both infusions (1). These results are in contrast to those observed in rats chronically fed cholesterol (4). In these rats there was a significant increase in the cholesterol content of an intermediate density lipoprotein fraction. The differences in the results between the two studies may reflect differences between the species used in the studies or the many other differences in experimental design.

In region II lymph lipoproteins, lymph LDL, the total cholesterol content and the proportion of total cholesterol in the esterified form increased with an increase in dietary cholesterol but the particle was still cholesterol-deficient and triglyceride-rich compared to the plasma LDL (Table 4). Although the slower migrating form of apoprotein B (apoB1), similar to that of plasma LDL, was predominant in lymph LDL, the faster migrating form of apoprotein B (apoB2) was also present. These results are consistent with the hypothesis that the intestine secretes an LDL-sized particle (31) that is present in lymph in addition to the LDL filtered into lymph from plasma.

Approximately 60% of the exogenous cholesterol in d > 1.006 g/ml lymph was transported by region III lymph lipoproteins (Fig. 1) which were similar in size to plasma HDL. High dietary cholesterol levels significantly increased the proportion of exogenous cholesterol in this fraction and exogenous cholesterol was incorporated preferentially as free cholesterol (Table 3). Although the apoprotein composition of the region III lipoprotein was similar to that of plasma HDL (Fig. 4), the lipid composition was significantly different (Table 4), in that the particles were phospholipid- and triglyceride-rich and deficient in free and esterified cholesterol in a manner similar to that for human lymph HDL (32). These findings are consistent with the hypothesis that the intestine secretes HDL, and the compositional data suggest that some discoidal or 'nascent' HDL may have been present as was found in the rat (33).

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