Particle size and composition of dog lymph chylomicrons

AKIRA YOKOYAMA* and D. B. ZILVERSMIT‡

Department of Physiology, University of Tennessee Medical Units, Memphis, Tennessee

SUMMARY Chyle from the thoracic duct of dogs fed cream or corn oil was fractionated into chylomicrons of different particle size: <140 mμ, 140–200 mμ, and >200 mμ. The composition of chylomicrons was studied before and after washing with saline solutions. In general the percentages of protein, phospholipid, cholesterol, and cholesterol ester were greater in the small chylomicrons than in the larger ones.

Washing the chylomicrons did not appear to affect their composition. Chylomicrons collected at different times during fat feeding showed significant differences in phospholipid and cholesterol concentrations. Storage of lymph at 4° was accompanied by alterations in phospholipid and cholesterol concentrations of the chylomicrons. The composition of chylomicrons is consistent with the hypothesis that at some stage during their formation the nontriglyceride components are present on the surface of the oil droplet.

KEY WORDS dog · lymph · chylomicrons · particle size · composition · triglyceride · phospholipid · cholesterol · protein · gradient centrifugation

THE COMPOSITION of the lipid particles present in lymph following the feeding of fat has been the subject of several investigations (1–8). However, the composition of chylomicrons of different sizes has not yet been determined.

The present communication reports analyses of chylomicrons of different sizes and after feeding cream or corn oil. The changes in chylomicron and lipoprotein composition during storage of the samples have also been studied.

*Postdoctoral Fellow. Present address: Department of Biochemistry, Sapporo Medical College, Sapporo, Japan.

‡Career Investigator of the American Heart Association.

EXPERIMENTAL METHODS

Preparation and Fractionation of Chylomicrons

Mongrel dogs (6–18 kg) of both sexes, fasted overnight, were fed, 2–3 hr before surgery, 60–100 ml of 36% fat in the form of whipping cream or corn oil emulsified in skim milk. The dogs were anesthetized with pentobarbital and a polyethylene cannula was inserted into the thoracic duct. Lymph was collected in an ice-cold erlenmeyer flask for 12–24 hr. During the collection of lymph, more whipping cream or corn oil emulsion was fed by stomach tube as needed to keep the lymph milky, and 0.5–1 ml of 0.9% saline per min was given intravenously by continuous drip.

The lymph was allowed to clot at 4°. The clot was removed by stirring and straining through thick gauze. To prepare washed chylomicrons the filtered lymph was centrifuged in a swinging bucket rotor at 25,000 rpm (90,000 × *g*, max) for ½–1 hr at 10°. After centrifugation, the packed fat layer at the top of the tube was removed with a spatula. The chylomicrons were suspended in 0.9% NaCl or phosphate buffer,[1] forced through a 20-gauge needle, and centrifuged under the conditions described above. This process of collecting, reemulsifying, and centrifuging was repeated three times. The washed chylomicrons were stored in a polyethylene container and were either fractionated immediately or stored in the dark at 4°. The washed chylomicrons were checked for the absence of soluble proteins by paper electrophoresis.

Chylomicrons of whole lymph or washed chylomicron preparations were separated into top, middle, and bottom fractions of different particle size by centrifugation in a sucrose gradient (9). Less than 8 mg of chylomicrons

[1] 5.84 g of NaCl, 2.22 g of Na_2HPO_4, 0.39 g of KH_2PO_4 per liter, pH = 7.4.

was centrifuged in 30-ml swinging buckets at 10,000 rpm for 1 hr at 25°. In some cases the fractions were washed by layering them under 10 ml of water and centrifuging again, this time at 25,000 rpm for 24 hr at 10°. This separated the chylomicrons from most of the sucrose solution. The tubes were sliced about 1.5 cm from the top and the chylomicron fraction was removed for chemical analysis.

Lipid Analyses

In order to determine the lipid and protein concentrations of the chylomicrons, we employed the extraction procedure of Hillyard et al. (4), in which ethanol–ether–chloroform 6:2:1 (v/v/v), ethanol–ether 3:1, and ether are used. The combined extracts were evaporated in vacuo at temperatures below 40°. To remove sucrose, a small amount of water was added to the residue, and lipids were reextracted with Skelly B (petroleum ether, bp 60–70°). The extract was dried with Na_2SO_4 and then evaporated as before. Lipids were reextracted with chloroform–methanol 2:1 (v/v) and washed by Folch's method to remove additional water-soluble constituents.

Lipids were fractionated by silicic acid chromatography (10). Each column was checked by thin-layer chromatography (TLC) in which the neutral lipids were developed with Skelly B–ethyl ether–glacial acetic acid 80:20:1 (11), and the phospholipids with chloroform–methanol–water 140:50:9.

Triglycerides were determined by periodate oxidation of glycerol and colorimetric determination of formaldehyde with chromotropic acid (12). Total, free, and esterified cholesterol were measured after saponification by the colorimetric method of Zak et al. (13). Phospholipid phosphorus was determined by the method of Bartlett (14) and protein nitrogen (on the residues left in the extraction procedure) by the Nessler method, as modified by Minari and Zilversmit (15).

The following empirical conversion factors were used in reporting lipid and protein data: cholesterol esters = 1.7 × (esterified cholesterol); phospholipid = 25 × (phospholipid phosphorus); protein = 6.25 × (nitrogen).

Tests of Extraction Procedure

The extraction and separation of lipids by conventional methods is made more difficult when large amounts of water-soluble constituents are present. In addition, the distribution of lipid classes in the chylomicron fraction differs greatly from that of serum or other tissues for which most of the column chromatographic procedures have been worked out. For this reason several extraction procedures were compared. One sample of washed chylomicrons obtained after cream feeding was extracted three times—once with 50, and twice with 20 volumes of Bloor's solvent (ethanol–diethyl ether 3:1). The solvent was heated to 65–70° for 5–10 min and after cooling and removal of the protein precipitate, the solvent was evaporated under reduced pressure. A second sample was extracted by the procedure of Hillyard et al. (4). After silicic acid column chromatography to remove the phospholipids, total cholesterol and glyceride glycerol were determined. Since cholesterol made up only about 1% of the total neutral lipid fraction, the completeness of the saponification was checked each time by TLC. Usually up to 2 mg lipid was completely saponified by 5 ml of 2% KOH in 95% ethanol (16). When more lipid was present or when saponification of cholesterol ester or triglyceride was found to be incomplete, 4% KOH was used. This increase in concentration did not affect the quantification of cholesterol by the $FeCl_3$–H_2SO_4 reagent.

The extraction with Hillyard and with Bloor solvents gave similar results: in one comparison we found for triglyceride, 37.6 and 36.1 mg/ml of chylomicrons and for cholesterol, 0.603 and 0.556 mg/ml respectively.

Recovery of Protein

The recovery of protein was checked in several ways. One sample of washed chylomicrons was divided into three portions and extracted by Hillyard or Bloor solvent as just described or with chloroform–methanol 2:1 as

TABLE 1 P^{32} AND C^{14} CONTENT OF CHYLOMICRONS

Rpm × Hr*	5,000 × 4				15,000 × 4				25,000 × 4			
	Lymph		Washed Chylomicrons		Lymph		Washed Chylomicrons		Lymph		Washed Chylomicrons	
Fraction	C^{14}	P^{32}/C^{14}	C^{14}	P^{32}/C^{14}	C^{14}	P^{32}/C^{14}	C^{14}	P^{32}/C^{14}	C^{14}	P^{32}/C^{14}	C^{14}	P^{32}/C^{14}
	%		%		%		%		%		%	
Top	7.8	0.16	67	0.61	64	0.37	94.9	0.79	85	0.46	94.6	0.72
Middle	15	0.23	14	1.03	15	0.75	1.91	—	3.8	1.30	2.08	3.31
Bottom	70	1.11	18	2.41	15	2.63	3.25	7.97	7.0	5.62	3.17	8.00
Below origin	6.9	2.97	—	—	5.6	4.36	—	—	4.0	4.13	—	—

* 5000 rpm for 4 hr represents about 6000 *g*-hr at the top and 14,000 *g*-hr at the bottom.

TABLE 2 LIPIDS AND PROTEIN OF CHYLOMICRONS FROM WHOLE LYMPH AFTER CREAM FEEDING

Particle Size	Total Lipid Distribution	TG*	PL*	Cholesterol			Protein‡
				Free*	Free†	Ester*	
mμ				%			
Top§ > 200	68.2	96.5	2.69	0.45	62.0	0.47	
	38.3	95.7	3.11	0.46	56.3	0.60	1.53
Middle§ 140–200	12.9	94.7	3.34	1.07	64.3	1.02	
	19.9	93.3	4.90	0.85	59.3	0.99	1.47
Bottom§ <140	15.2	85.7	9.12	1.49	41.1	3.65	
	36.7	88.0	9.23	1.10	54.4	1.57	9.95
Below origin§	3.44	53.9	24.3	3.98	27.5	17.9	
	5.00	81.2	12.5	2.12	46.0	4.23	43.7

* Percentage of total lipid.
† Percentage of total cholesterol.
‡ Percentage of total weight.
§ Samples from two different animals. Top line: sample in which entire fraction was analyzed. Bottom line: sample in which chylomicrons were analyzed after additional water wash (see text). This difference in method affected only the "below origin" fraction, in which soluble lipoproteins were present.

described by Folch et al. (17). To the three precipitates were added 5 ml of 5% trichloroacetic acid (TCA) and 5 ml of diethyl ether. After centrifugation the precipitate was washed twice more with diethyl ether. The residues were dissolved in KOH, and nitrogen was determined by the Kjeldahl procedure (15). The nitrogen contents were 330, 319, and 325 μg respectively. In addition, the lipid extracts were tested for presence of protein in the following manner. The chloroform–methanol extract was evaporated, and the residue washed three times with petroleum ether, once with 5% TCA, and three times with diethyl ether. By the Kjeldahl method 16 μg of nitrogen was found which, if this were protein, would represent 5% of the total protein. Bloor and Hillyard extracts were dialyzed in Visking cellulose casing against chloroform–methanol at room temperature or at reflux temperature in a Soxhlet apparatus until no more lipid could be removed. No protein was detected inside the bag by Kjeldahl nitrogen determination or by spot test with tetrabromophenolphthalein ethyl ester (18). Although the three extraction procedures gave essentially the same results for protein nitrogen, the chloroform–methanol extraction was judged least convenient since packing of the precipitate in the high density solvent was difficult.

RESULTS

Doubly Labeled Chylomicrons

The first indication that chemical composition of chylomicrons might be related to particle size appeared when doubly labeled chylomicrons were isolated from the thoracic duct lymph of a dog fed cream containing phosphate-P^{32} and palmitate-C^{14}. Table 1 shows the results of this experiment. Three aliquots of defibrinated lymph or washed chylomicrons were centrifuged in sucrose gradients at three different speeds. The four fractions obtained from the centrifuge tube were counted for C^{14} and P^{32} in a two-channel liquid scintillation counter. Column chromatography showed that less than 10% of the C^{14} was incorporated into the combined cholesterol ester and phospholipid fraction,[2] so that the ratio P^{32}/C^{14} reflects the ratio of phospholipid to triglyceride if one can assume that the specific activity of these lipid fractions is independent of particle size.

As seen from Table 1 this ratio in the top and bottom fractions differed considerably, indicating that relatively more phospholipid was present in the small than in the large chylomicrons. This was true not only for whole lymph, which presumably contained some soluble lipoproteins, particularly in the lower layers of the tube, but also for washed chylomicrons, which were free from soluble lipoproteins. The P^{32}/C^{14} ratio for the latter chylomicrons varied from 0.61 at the top to 8.00 at the bottom.

Lipid Composition and Particle Size

Tables 2–5 present the lipid and protein composition of lymph chylomicrons after feeding cream or corn oil. Both types of chylomicrons were studied in defibrinated lymph as well as in washed chylomicron preparations. The washed chylomicrons were derived from the lymph samples referred to in the preceding table. The "top," "middle," and "bottom" represent three layers in the sucrose gradient above the origin. In the whole lymph (Tables 2 and 4) a fourth fraction below the origin was analyzed. In one sample of chyle (Table 2) more than two-thirds of the particles were larger than 200 mμ (top layer), whereas in the second sample only 38% of the lipid was found in the top layer. Washing the

[2] B. Edgren and D. B. Zilversmit, unpublished observations.

TABLE 3 LIPIDS AND PROTEIN OF WASHED CREAM CHYLOMICRONS

Particle Size	Total Lipid Distribution	TG*	PL*	Cholesterol			Protein‡
				Free*	Free†	Ester*	
mμ			%				
Top§ >200	87.0	95.9	3.16	0.53	64.3	0.50	
	68.4	95.3	3.77	0.55	67.2	0.46	0.59
Middle§ 140–200	8.34	94.4	3.33	1.70	78.4	0.79	
	18.40	92.0	6.31	0.94	68.8	0.73	1.81
Bottom§ <140	4.81	94.6	2.36	2.15	80.7	0.87	
	13.30	88.3	9.14	1.45	68.3	1.15	2.26

* Percentage of total lipid.
† Percentage of total cholesterol.
‡ Percentage of total weight.
§ Samples from the same two animals as in Table 2.

chylomicrons shifted the particle size distribution so that a larger fraction of the total lipid, 87 and 68% respectively, was found in the top layer. This shift was probably the result of aggregation or coalescence of chylomicrons but could have been due, in part at least, to the preferential loss of small chylomicrons in the washing procedure. Observations by dark field and phase contrast microscopy of wet preparations confirmed the tendency of washed chylomicrons to form aggregates and also showed that washed chylomicrons stick more readily to the glass slide and cover slip than the chylomicrons of native chyle.

The lipid composition of chylomicrons in the three size classes presented in Tables 2–5 varied over a wide range. In all but one instance the smaller particles showed appreciably higher proportions of phospholipid and of free and esterified cholesterol. Also the protein concentration in the smaller chylomicrons was greater than that of the larger particles. Since the original chyle contains considerable amounts of soluble proteins, the protein content of the chylomicrons in lymph, particularly in the smaller chylomicron fractions, probably reflects some contamination with soluble protein. The protein content of the largest chylomicrons was only about 0.5%, whereas that of the smaller chylomicrons (Tables 3 and 5) is >2%.

It should be noted that the data in Tables 2 and 3 are derived from experiments with chyle from two different animals. The upper values of the pair given for each fraction were obtained by extracting the layers from the sucrose-gradient tube with Hillyard's solvent directly. The data on the lower lines of Tables 2 and 3, and the data in Tables 4 and 5, were obtained by extraction after a second centrifugation and water wash as described in the Experimental Methods section. The removal of soluble lipoproteins by the second centrifugation probably accounts for the much higher percentage of triglyceride found in the "below origin" fraction of the second sample in Table 2.

In each experiment the amounts of the several lipid classes and of protein in each fraction were added and compared with the corresponding fractions present in the original lymph or washed chylomicrons. The recoveries were, on the whole, close to 100%. The lowest recovery was 89% for the protein in Table 2; the highest was 113% for phospholipid in Table 4.

Effect of Cold Storage and Collection Time

While the analyses reported here were being performed, certain discrepancies were observed when chylomicrons from a chyle sample kept at 4° for several days were reanalyzed. In addition, it was observed that chylomi-

TABLE 4 LIPIDS AND PROTEIN OF LYMPH CHYLOMICRONS AFTER CORN OIL FEEDING

Particle Size	Total Lipid Distribution	TG*	PL*	Cholesterol			Protein‡
				Free*	Free†	Ester*	
mμ			%				
Top >200	63.2	95.0	4.29	0.52	72.3	0.34	0.52
Middle 140–200	15.9	84.0	7.80	—	—	—	1.58
Bottom <140	18.5	85.3	12.5	1.00	56.0	1.34	5.90
Below origin	1.97	51.8	41.3	2.27	44.8	4.77	40.4

* Percentage of total lipid.
† Percentage of total cholesterol.
‡ Percentage of total weight.

TABLE 5 LIPIDS AND PROTEIN OF WASHED CORN OIL CHYLOMICRONS

Particle Size	Total Lipid Distribution	TG*	PL*	Cholesterol			Protein‡
				Free*	Free†	Ester*	
mμ				%			
Top >200	71.7	96.4	2.83	0.41	74.2	0.24	0.52
Middle 140–200	17.1	94.4	4.67	0.68	79.5	0.30	2.79§
Bottom <140	11.1	88.4	9.77	0.98	67.7	0.79	2.02

* Percentage of total lipid.
† Percentage of total cholesterol.
‡ Percentage of total weight.
§ This value is higher than that of the bottom fraction and of the middle fraction in Table 4. This may be an analytical error; no other explanation can be offered.

crons collected at different intervals after fat absorption showed different compositions. To study this phenomenon more closely, lymph was collected from a dog fed cream. Lymph was collected for three periods, one sample immediately after cannulation for about 2 hr and two samples during subsequent periods up to 10 hr. The first batch was divided into three portions. One portion was centrifuged immediately and the chylomicrons were washed as described before. The other two portions were stored in the refrigerator at 4° for 3 and 7 days respectively. After storage, the chylomicrons were washed and analyzed as before. The chylomicrons from the chyle collected between 2 and 10 hr were kept refrigerated for 10–12 hr prior to washing and analysis.

Table 6 shows that differences in composition were observed in samples collected from the thoracic duct at different times and in samples kept in the refrigerator for up to 7 days. In this particular experiment the phospholipid content of the chylomicrons increased from 5.7 to 6.5% after 7 days at 4°. In a previous similar experiment the increase was from 5.2 to 6.2%. Although storage did not change the total cholesterol content of the chylomicrons significantly, an increase from 1.8 to 2.6% was observed in a previous experiment. More pronounced are differences in the composition of chylomicrons collected at different time intervals after cannulation and fat feeding. Thus the cholesterol of chylomicrons collected 0–4 hr after cannulation of the thoracic duct was 1.9% as compared to 0.74% in chylomicrons collected during the next 6 hr. In a similar experiment the chylomicrons collected first contained 1.83% cholesterol, whereas those collected later had a cholesterol content of 0.73%. These differences are not always in the same direction; in another experiment with chylomicrons after corn oil feeding, the cholesterol content of chylomicrons collected early was 0.83%, whereas that of the later ones was 1.51%.

DISCUSSION

The composition of lymph chylomicrons has been measured by various investigators, but little or no attention has been paid to the relation of composition to particle size. The present study shows that there is a definite relation of particle size to lipid composition. The increase in the relative amounts of protein, phospholipid, and cholesterol in the smaller particles makes it reasonable to look upon chylomicrons as lipoproteins with variable triglyceride content.

The data presented in this paper also make it possible to draw some inferences about chylomicron structure. If one assumes that a shell of protein of constant thickness covers lipid particles of varying size, the protein–lipid ratio would be inversely proportional to the particle

TABLE 6 EFFECT OF COLLECTION INTERVAL AND STORAGE OF LYMPH ON CHYLOMICRON COMPOSITION

Time After Cannulation	Storage at 4°	Total Lipid Content of Lymph	Phospholipid*	Cholesterol		Protein‡
				Total*	Free†	
hr	days	wt %		%		
0–2	0	2.3	5.70	1.89	44	1.83
	3	2.3	6.18	1.95	46	1.81
	7	2.3	6.53	1.97	47	1.77
2–4	0.5	1.1	6.71	1.94	67	2.97
4–10	0.5	4.5	5.72	0.74	70	1.22

* Percentage of total lipid.
† Percentage of total cholesterol.
‡ Percentage of total weight.

diameter. If, on the other hand, one postulates that chylomicrons of different size have a constant protein core with varying amounts of lipid, the protein–lipid ratio would vary inversely with the cube of the diameter.

From gradient centrifugation one may estimate that the average particle size in the bottom fraction is about 100 mμ; in the top fraction it is unlikely to exceed 300 to 400 mμ (9). The percentage of protein in the larger particles is about one-fourth that of the smaller ones (Tables 3 and 5), which is consistent with the hypothesis that the protein is present at the oil–water interface. The larger percentages of phospholipid and of free and esterified cholesterol in the smaller chylomicrons might also indicate that these lipids are present at the interface. Other experiments (19) have suggested, however, that the cholesterol ester and part of the free cholesterol are dissolved in the oil phase. A combination of these observations would suggest that chylomicrons are initially formed from a triglyceride droplet, with lipoprotein added to the interface in the intestinal mucosa or possibly upon extrusion into the lymphatics. The deformation, during the addition, of the lipoprotein at the oil–water interface might release the cholesterol ester and part of the unesterified cholesterol, as was observed in thin films at the heptane–water interface (20). Such a view would explain why more cholesterol ester is present in the smaller particles even though ultimately it is found dissolved in the oil phase.

A secondary consideration of these studies was to find the effect, if any, of dietary fat composition on the chemical constitution of chylomicrons. Jones et al. (21) have reported that rats fed butter fat formed "giant chylomicrons" in contrast to rats fed oil. In our own studies we were unable to discover any marked differences in lipid composition between one batch of corn oil chylomicrons and two collections of cream chylomicrons. Nor were any "giant chylomicrons" observed in the latter preparations when viewed by phase contrast or dark field microscopy.

If small differences in lipid class composition should exist between chylomicrons from dogs fed cream or corn oil, such differences might be obscured by the changes taking place in the chylomicrons during cold storage. These differences may in part be due to unequal exchanges of lipids between chylomicrons and the aqueous phase of lymph similar to those observed when washed lymph chylomicrons are incubated with serum (10). Under these conditions chylomicrons increased their free cholesterol content but lost phospholipids to the serum lipoproteins. The extent of these transfers was found to depend markedly on the relative concentrations of chylomicrons and serum during incubation. A similar sequence of reactions may account for the differences in composition of chylomicrons collected from the same dog at different times after cannulation. In the anesthetized animal the lipid concentration of thoracic duct lymph is quite variable. During periods of active fat transport the lymph is quite turbid. In analogy with the behavior of lymph chylomicrons when incubated with serum (10), one would expect that in concentrated lymph the relative amount of lipid transfer between chylomicrons and the lipoproteins of lymph would be less than when there are fewer chylomicrons per milliliter of lymph.

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References

1. Laurell, C. B. *Acta Physiol. Scand.* **30:** 289, 1954.
2. Robinson, D. S. *Quart. J. Exptl. Physiol.* **40:** 112, 1955.
3. Havel, R. J., and D. S. Fredrickson. *J. Clin. Invest.* **35:** 1025, 1956.
4. Hillyard, L. A., I. L. Chaikoff, C. Entenman, and W. O. Reinhardt. *J. Biol. Chem.* **233:** 838, 1958.
5. Savary, P., M. J. Constantin, and P. Desnuelle. *Biochim. Biophys. Acta* **48:** 562, 1961.
6. Dole, V. P., and J. T. Hamlin III. *Physiol. Rev.* **42:** 674, 1962.
7. Bierman, E. L., E. Gordis, and J. T. Hamlin III. *J. Clin. Invest.* **41:** 2254, 1962.
8. Seaman, G. V. F., and R. L. Swank. *J. Physiol.* **168:** 118, 1963.
9. Pinter, G. G., and D. B. Zilversmit. *Biochim. Biophys. Acta* **59:** 116, 1962.
10. Minari, O., and D. B. Zilversmit. *J. Lipid Res.* **4:** 424, 1963.
11. Wood, P., K. Imaichi, J. Knowles, G. Michaels, and L. Kinsell. *J. Lipid Res.* **5:** 225, 1964.
12. Newman, H. A. I., Ching-Tong Liu, and D. B. Zilversmit. *J. Lipid Res.* **2:** 403, 1961.
13. Zak, B., N. Moss, A. J. Boyle, and A. Zlatkis. *Anal. Chem.* **26:** 776, 1954.
14. Bartlett, G. R. *J. Biol. Chem.* **234:** 466, 1959.
15. Minari, O., and D. B. Zilversmit. *Anal. Biochem.* **6:** 320, 1963.
16. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. *J. Biol. Chem.* **195:** 357, 1952.
17. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.
18. Feigl, F. *Spot Tests in Organic Analysis.* Elsevier Publ. Co., Amsterdam, 1960, p. 441.
19. Zilversmit, D. B. *Federation Proc.* **23:** 501, 1964.
20. Zilversmit, D. B. *J. Lipid Res.* **5:** 300, 1964.
21. Jones, R., W. A. Thomas, and R. F. Scott. *Exptl. Molec. Path.* **1:** 65, 1962.