Lipoproteins and apolipoproteins in intestinal lymph of the preruminant calf, *Bos* spp., at peak lipid absorption

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Abstract We have recently evaluated the in vivo role of the liver in lipoprotein homeostasis in the preruminant calf (Bauchart, D., D. Durand, P. M. Laplaud, P. Forgez, S. Goulinet, and M. J. Chapman, 1989. *J. Lipid Res.* **30:** 1499–1514). We now present the partial characterization of lipoprotein particles in postprandial intestinal lymph at peak lipid absorption (i.e., 10 h after a meal) in the preruminant calf fed a curdled milk replacer. Intestinal lymph from four male preruminant calves was analyzed for its content of lipids and fractionated by sequential and density gradient ultracentrifugation into chylomicrons ($S_f > 400$), very low density lipoproteins (VLDL) ($S_f < 400$; $d < 1.006$ g/ml), and a series of lipoprotein subfractions with $d > 1.006$ g/ml. Postprandial lymph contained predominantly triglycerides (1099 $\pm$ 611 mg/100 ml), with lesser amounts of phospholipids (197 $\pm$ 107 mg/100 ml) and cholesterol (52 $\pm$ 30 mg/100 ml). The most abundant particles were triglyceride-rich chylomicrons and VLDL which accounted for $\approx 76\%$ and $\approx 19\%$, respectively, of total $d < 1.21$ g/ml lipoproteins. As judged by negative stain electron microscopy, chylomicron particle diameters ranged from 650 to 2400 Å, while VLDL were smaller and distributed over a distinct size range (340–860 Å). These two lipoprotein classes each presented protein components with M_r comparable to those of human apoB-48, apoA-I, and C apoproteins, together with an M_r 52,000 protein resembling human β2-glycoprotein-I. In addition, VLDL exhibited a polypeptide with $M_r \approx 61,000$. Lymph lipoproteins with $d > 1.006$ g/ml consisted primarily ($\approx 81\%$ of total) of particles distributed over the 1.053–1.119 g/ml density range. Electrophoretic analysis of the latter lipoprotein fraction showed it to be heterogeneous, including particles with the migration characteristics of low and of high density lipoproteins, respectively. Subfractions in the d 1.053–1.076 g/ml range were dominated by particles with Stokes diameters typical of high density lipoproteins (HDL), but also contained three different populations of low density lipoprotein-like particles. The high molecular weight apolipoproteins in these same cholesteryl ester-rich ($>30\%$ of lipoprotein mass) subfractions comprised components with M_r resembling those of human apoB-100 and apoB-48, respectively, and with the latter protein predominating to a varying degree. A counterpart to human apoA-I was the major protein component over the entire density range from d 1.053 to 1.119 g/ml. Minor amounts of lipoproteins (<10 mg/ml) were present at higher densities (up to d 1.180 g/ml); in contrast to HDL of d 1.053–1.119 g/ml, the proportion of cholesteryl esters in these particles decreased with density; as in plasma however, their triglyceride content increased with density to attain 8% in the subfraction of highest density. ■ Thus, postprandial lymph in the preruminant calf contains a spectrum of lipoprotein particles that are comparable in their physicochemical properties to lymph lipoproteins as observed in the rat and in humans. The size and physiology of the calf allow large volume samplings of blood and lymph and relatively easy access to intestinal blood and lymph vessels. Our data suggest that the preruminant calf constitutes a valuable model for studies of intestinal lipoprotein metabolism. — **Laplaud, P. M., D. Bauchart, D. Durand, and M. J. Chapman.** Lipoproteins and apolipoproteins in intestinal lymph of the preruminant calf, *Bos* spp., at peak lipid absorption. *J. Lipid Res.* 1990. **31:** 1781–1792.

Supplementary key words intestinal lymph duct • cannulation in vivo • chylomicrons • VLDL • density gradient ultracentrifugation • gradient gel electrophoresis • electron microscopy

The digestive physiology of adult bovine species is characterized by the possession of a rumen and involves complex fermentation processes that precede lipid absorption. The digestive system of the postnatal calf is, however, distinct from that of the adult bovine. Thus, from birth to the age of 1–2 months, the reticulorumen of the calf is poorly developed. Milk, the sole dietary constituent, induces a closing reflex of the esophageal groove, thereby allowing direct passage of digested milk to the forestomachs. This digestive feature is characterized by the absence of fermentation processes. No microbial modification of the chemical structure of dietary lipids occurs. On this basis, the calf, a so-called preruminant, may

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Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins, with densities as defined; apo, apolipoprotein; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; BW, body weight; TC, total cholesterol; FC, free cholesterol; CE, cholesteryl ester; PL, phospholipids; CETP, cholesteryl ester transfer protein.

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be considered a transient, functional monogastric. From a practical viewpoint, blood vessels afferent to, or efferent from, the liver are easily accessible in the calf, and large volumes of plasma can be drawn without undue stress to the animal (1). Thus, the calf presents as a highly suitable model for studies of lipoprotein metabolism and, indeed, has allowed us to evaluate mechanisms of lipid absorption, transport, and utilization (2–6).

In a recent report (7), we described the characterization of the lipoproteins and apolipoproteins of fasting calf plasma obtained at three sites of sampling, i.e., portal vein, hepatic artery, and hepatic vein. We reported, for the first time, the in vivo determination of lipoprotein balance across the liver in the calf, thus demonstrating that the hepatic HDL flux showed a net production of light HDL (d 1.060–1.091 g/ml), together with a net uptake of denser HDL (d 1.091–1.180 g/ml). In addition, our data revealed the presence in the portal vein of 3- to 6-fold higher levels of VLDL as compared to those found in hepatic artery or hepatic vein. These findings suggested a direct secretion of intestinal VLDL into the portal vein and highlighted the potential interest of further investigations aimed at exploring intestinal lipoprotein metabolism in the calf. In an initial step, we undertook the characterization of the lipoprotein particles in intestinal lymph at peak lipid absorption, and we describe these studies in the present report.

MATERIALS AND METHODS

Animals and diets

Four crossbred Friesian-Holstein male calves were used for the present experiments. These animals were provided by the Laboratory of Research on Lactation (INRA, Theix, France). They were housed individually in wooden stalls on a litter of wood shavings in an air-conditioned room (average temperature: 20°C, relative humidity: 88%).

Liquid milk replacer was bucket-fed in two equal meals per day (09.00 h and 16.00 h), and contained 16% dry matter (gross energy, 5.182 kcal/g) which was composed of 68% spray-dried skim milk powder (i.e., 22.8 weight % protein), 23% tallow, 6.8% corn starch, and 2.2% vitamin and mineral mixture (Roquette frères, Lestrem, France). The total lipid and fatty acid content of milk powder amounted to 24.1% and 22.6% of the dry matter, respectively; the principal fatty acids were palmitic (27.6% of total), stearic (18.6%), oleic (35.1%), and linoleic (3.5%).

At the time of operation and sampling, the calves were 16 ± 5 days old (mean ± SD), their body weights were 46 ± 4 kg, and they exhibited a growth rate of 609 ± 28 g/day for a dry matter intake amounting to 60 g/kg $BW^{0.75}$. The day before the experiment was initiated, the calves received their meal at 11 PM, and surgical intervention was performed on the next morning at 9 AM, i.e., 10 h after the meal. This time interval was chosen on the basis of the data of Romsos and McGilliard (8), who demonstrated that maximum lipid absorption in the calf occurred approximately 10 h after feeding.

Surgical technique

A catheter was introduced in the main intestinal lymph duct according to the technique of Romsos and McGilliard (9). Briefly, the lymph duct was carefully dissected free, and two ligatures were passed around it. The caudal ligature was tied. A short longitudinal incision was made in the duct between the two ligatures and a cannula was directed into the duct for 2 to 4 cm. The other ligature was subsequently tied around the duct and cannula.

Lymph samples

The volume of intestinal lymph thus obtained was approximately 200 ml. Sodium-EDTA (final concentration 1 mM) and sodium azide (0.01%, w/v) were immediately added, and lymph was stored at 4°C until lipoprotein fractionation began, i.e., typically within 24 to 48 h of surgery.

Chemical analysis of lymph lipids and of the lipid and protein content of lymph lipoproteins

Total cholesterol (TC) and free cholesterol (FC) were measured enzymatically using the reagent kit supplied by Merck (CHOD-iodide, Merkotest No. 14350, Darmstadt, West Germany). Cholesteryl ester (CE) content was calculated using the relation CE = (TC – FC) × 1.68. Triglyceride was estimated by the enzymatic method of Fossati and Prencipe (10), using Biomérieux reagent kit (PAP 1000, No. 6.123.6; Biomérieux, Charbonnières-les-Bains, France) which determines total glycerol content. Therefore, the term triglyceride designates the plasma content of triglycerides plus free glycerol, while the content of triglycerides in the various lipoprotein subfractions corresponds specifically to triglyceride since the latter were dialyzed to remove free glycerol (11).

Quantitation of phospholipids (PL) (as phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin) was determined by the enzymatic method of Trinder as described by Takayama et al. (12). This procedure measures choline liberated after hydrolysis by phospholipase D and involves use of the Biomérieux kit (PAP 150, No. 6.149.1), and therefore underestimates the total content of phospholipid in each lipoprotein fraction. It may be added, however, that our analyses of the phospholipid content of whole intestinal lymph in the calf suggest that choline-containing phospholipids represent up to ≈90% of the total (D. Bauchart, unpublished results). These data are consistent with those on plasma lipoprotein phospholipids in calves of similar age (13).

Protein concentrations were determined in each lipoprotein fraction by the procedure of Lowry et al. using 0.5 M sodium desoxycholate (Fluka, Switzerland) and using bovine serum albumin as the working standard (14).

Lipoprotein isolation

The different ultracentrifugation steps used for lipoprotein fractionation were conducted in a Kontron model Centrikon T-2060 ultracentrifuge using a TST 41-14 swinging bucket rotor, with the exception of the separation of chylomicrons and VLDL (see below). Five ml of total lymph was first placed in the bottom of each ultracentrifuge tube and layered with 7 ml of 0.15 M NaCl, d 1.006 g/ml. These tubes were then centrifuged for 16 h at 40,000 rpm and 15°C. The top 2-ml fraction (fraction I) contained both chylomicrons and VLDL. An intermediate fraction of 5 ml (fraction II) had been shown in preliminary experiments to contain no measurable amounts of protein and/or lipid and was thus discarded. The bottom 5-ml fraction (fraction III) contained all lipoproteins with d > 1.006 g/ml.

Fraction I obtained as above was subsequently used for separation of chylomicrons ($S_f > 400$) from VLDL ($S_f < 400$; d < 1.006 g/ml). This separation was performed in an MSE Prepspin 50 ultracentrifuge, equipped with an MSE 6 × 14 ml titanium swing-out rotor. For this purpose, 4 ml of fraction I material was placed in the bottom of each tube, and overlayered with 8 ml of distilled water containing EDTA (1 mM) and sodium azide (0.02%). Centrifugation was then performed for 24 min at 28,000 rpm and 15°C. This resulted in the collection of chylomicrons, defined as particles of $S_f > 400$, in the top 6 ml. The bottom 6 ml was recovered separately, and recentrifuged for 1 h under the same conditions. After this latter centrifugal run, the top 1-ml fraction contained only traces of lipoprotein material (usually 1–2%, expressed as protein, of total lipoproteins with d < 1.006 g/ml). According to its flotation characteristics, this material should contain traces of both small chylomicrons (S_f slightly higher than 400) and large VLDL (S_f slightly lower than 400). The presence of these two lipoprotein species was verified by lipoprotein electrophoresis on Lipofilm polyacrylamide gel (see below; electrophoretic methods) after suitable concentration in an Amicon 8MC ultrafiltration system fitted with XM 50 ultrafiltration membranes. Such material was ultimately discarded. The bottom 5 ml in the centrifugation tube contained pure VLDL ($S_f < 400$; d < 1.006 g/ml) which were used for subsequent analyses.

Fraction III was subjected to density gradient ultracentrifugation in order to subfractionate lipoproteins with d > 1.006 g/ml. Discontinuous gradients including five steps with respective densities 1.24 g/ml, 1.21 g/ml (lymph fraction III-KBr), 1.063 g/ml, 1.019 g/ml, and 1.006 g/ml were thus constructed according to the methodology of

Chapman et al. (15) as described in our previous report (7). Such gradients were subsequently centrifuged for 46 h at 39,000 rpm and 15°C. Twenty-two successive fractions of 0.4 ml each (0.5 ml for the top fraction) were then recovered and dialyzed against 0.05 M NaCl, 0.005 M NH$_4$HCO$_3$, 0.04% Na$_2$-EDTA, 0.01% sodium azide, and 0.001% sodium merthiolate, pH 7.4, as previously described (7).

Electrophoretic methods

Polyacrylamide gel electrophoresis of lymph lipoprotein fractions was performed using commercially available polyacrylamide gel slabs (Lipofilm, Sebia, Issy-les-Moulineaux, France). Lipofilm slabs are constructed to give a discontinuous gradient from 2% (at point of sample application) to 3% (running gel). In this system, chylomicrons remain at the origin, whereas VLDL migrate to the junction of the two gels.

Evaluation of particle diameter and the potential heterogeneity of lipoprotein size species was performed on lymph fractions by electrophoresis at 15°C on continuous polyacrylamide gradient gel slabs from 4 to 30% (PAA 4/30 Pharmacia, Uppsala, Sweden). Gels were stained with 0.5% Coomassie Brilliant blue (Sigma) and destained in 10% trichloroacetic acid. No attempt was made to quantify the bands detected in these electrophoreses. The Stokes diameters of lipoprotein particles were determined using the Stokes-Einstein equation (16) from a calibration curve constructed from a series of protein markers (Pharmacia) i.e., 71 Å (bovine serum albumin), 81 Å (lactic dehydrogenase), 104 Å (catalase), 122 Å (ferritin), and 170 Å (thyroglobulin) (17).

Prior to electrophoretic examination of the content of their apolipoprotein moiety, lipoproteins were delipidated with ethanol–diethylether 3:1 (v/v) as described by Brown, Levy, and Fredrickson (18); the apoprotein residue was dried under N$_2$. The molecular weights of apolipoproteins were estimated by electrophoresis in SDS-polyacrylamide gels of either 10% monomer concentration as described by Weber and Osborn (19), or 3% monomer concentration according to the modification of Weisgraber et al. (20) of the methodology of Stephens (21). Calibration curves for estimation of molecular weights were constructed using Low and High Molecular Weight electrophoresis calibration kits from Pharmacia Fine Chemicals. Staining of the gels was performed using either Coomassie Brilliant blue R-250 for 10% monomer gels, or the technique of Karlson et al. (22) for 3% monomer gels.

Electron microscopic studies

Preparations of lymph chylomicrons and VLDL, which contained less than 0.1 mg protein/ml, were negatively stained with 2% potassium phosphotungstate at pH 7.4. Approximately 100 µl of each lipoprotein preparation was

applied to a Formvar-carbon-coated nickel grid, and the excess was adsorbed onto filter paper. After drying in air for 1 min, a similar volume of stain solution was applied; the excess stain was removed by blotting as before, and the grid was allowed to dry for 5–10 min.

The stained preparations were examined in a Philips CM10 electron microscope; microscope magnification was precalibrated by use of a germanium-shadowed carbon replica of a ruled diffraction grating bearing 54,864 lines per inch. Three separate preparations of each lipoprotein fraction were typically examined; a minimum of 100 particles was measured on each of a series of 5 to 10 photomicrographs by use of a micro-comparator. The range in original magnification of such micrographs was between 10,000 × and 50,000 × .

RESULTS

Lipid content of intestinal lymph

As shown in **Table 1**, our data on the relative concentrations of the different lipid classes in intestinal lymph provide evidence for the prominence of triglycerides (mean ± SD: 1099 ± 611 mg/100 ml) over both phospholipids (197 ± 107 mg/100 ml) and cholesterol (52 ± 30 mg/100 ml). The concentration of esterified cholesterol was slightly greater than that of free cholesterol, thus leading to a mean value of 0.54 for the ratio of the concentration of esterified cholesterol/total cholesterol. However, the high values for the standard deviations attest to the fact that, despite strict adhesion to reproducible experimental conditions, large discrepancies were evident between the minimal and maximal concentrations of the different lymph lipids in the four calves that were examined. Therefore, we have included such maximum and minimum values in Table 1.

Data on the weight percent distribution of the different lipid constituents in the same lymph samples are also presented in Table 1. In contrast with the results reported above, standard deviations in this second part of the table are compatible with a relatively homogeneous lipid composition of intestinal lymph in the four calves. Indeed, triglycerides consistently represented approximately 80% of total lipids (mean ± SD = 80.4 ± 4.0%), while phospholipids accounted for approximately 15%. Cholesterol was therefore a quantitatively minor component, representing less than 5% of total lipids.

Characterization of intestinal lymph lipoproteins

As demonstrated by the results reported herein, the density spectrum of intestinal lymph lipoproteins, when obtained during the period of postprandial maximum lipid absorption, was dominated by particles with d < 1.006 g/ml, among which chylomicrons predominated. We were therefore led to initially float all such particles together and to subsequently separate chylomicrons from VLDL in this fraction (see Materials and Methods). By contrast, the bottom fraction from the first ultracentrifugal step was taken for analysis of the density profile of lipoproteins with d > 1.006 g/ml by gradient ultracentrifugation. In an attempt to facilitate direct comparison with our previous results dealing with calf plasma lipoproteins (7), we used, at this stage, the same isopycnic density gradient ultracentrifugal procedure, thereby allowing analysis of lipoprotein particles with hydrated densities up to 1.180 g/ml. Furthermore, we maintained the same nomenclature for lipoprotein fractions as in our preceding report (7), thus defining LDL as lipoproteins of d 1.026 to 1.060 g/ml, light HDL (HDL$_L$) as lipoproteins of d 1.060 to 1.091 g/ml, and heavy HDL (HDL$_H$) as lipoproteins of d 1.091 to 1.180 g/ml.

Table 2 presents results on both the chemical compositions and concentrations of calf lymph lipoproteins. With regard to the latter parameter, our data provide evidence for the quantitative predominance of chylomicrons and to a lesser degree, of VLDL particles, which accounted, respectively, for 75.3 ± 2.7% and 19.8 ± 3.8% (means ± SD) of total lymph lipoproteins. Lipoproteins of higher density were essentially represented by particles distributing in the d 1.053–1.119 g/ml range, which accounted for ≈81% (mean) of lipoproteins with d > 1.006 g/ml. It

TABLE 1. Lipid constituents of intestinal lymph in the preruminant calf

	Concentration[a]			Weight % Distribution
	Mean ± SD	Minimal	Maximal	
	mg/dl			*mean ± SD*
Triglycerides	1099 ± 611	472	1267	80.4 ± 4.0
Phospholipids	197 ± 107	92	366	14.4 ± 2.7
Cholesterol				
Total[b]	52 ± 30	22	94	
Free	24 ± 11	10	40	1.8 ± 1.5
Cholesteryl esters	48 ± 27	20	90	3.5 ± 1.1

[a]Each analysis was performed in duplicate on lymph samples from each of four calves.
[b]Values for total cholesterol represent the sum of free and esterified cholesterol.

TABLE 2. Mean percentage (weight) chemical composition of lipoprotein subfractions isolated from intestinal lymph obtained at peak lipid absorption in preruminant calves[a]

Component	Chylomicrons[c]	VLDL[c]	LDL							HDL_L							HDL_H			
Density Gradient Fractions and Subfractions[b]			1–5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Density limits (g/ml)	<1.026	<1.026	<1.026	1.026–1.030	1.030–1.035	1.035–1.040	1.040–1.046	1.046–1.053	1.053–1.060	1.060–1.068	1.068–1.076	1.076–1.086	1.086–1.091	1.091–1.108	1.108–1.119	1.119–1.131	1.131–1.143	1.143–1.154	1.154–1.167	1.167–1.180
Free cholesterol	1.1[d]	0.6	ND[f]	6.7	5.8	5.2	7.2	5.3	3.8	2.2	1.6	1.5	1.3	1.7	2.0	3.0	4.1	5.2	7.1	7.8
Cholesteryl ester	0.9	1.3	ND	16.0	18.0	20.7	22.2	26.4	31.5	31.6	32.8	30.5	28.2	26.9	25.0	21.1	14.8	10.8	3.5	1.1
Triglyceride	87.8	77.7	ND	35.1	29.3	26.3	21.7	18.5	5.4	6.9	3.8	3.6	3.7	3.9	4.5	4.8	5.7	7.6	7.6	8.4
Phospholipid	8.1	12.2	ND	22.3	23.8	22.6	19.8	22.5	32.5	28.4	28.6	28.5	27.7	26.7	22.3	24.5	25.2	19.0	14.3	9.2
Protein	2.0	8.2	ND	19.9	23.1	25.2	29.1	27.3	26.9	30.9	33.2	35.9	39.0	40.8	46.2	46.7	50.2	57.4	67.6	73.5
Lipoprotein concentration (mg/dl)[e]	1099.6 ±313.8	276.5 ±20.5		0.7 ±0.1	1.0 ±0.2	0.8 ±0.2	1.1 ±0.2	1.5 ±0.4	4.4 ±0.8	7.9 ±3.6	13.1 ±8.1	12.6 ±5.6	9.0 ±4.7	8.3 ±4.6	6.5 ±2.5	3.1 ±1.6	2.1 ±0.8	1.5 ±0.4	1.3 ±0.4	1.7 ±0.8
Mean % of lipoproteins with d > 1.006 g/ml				0.9	1.3	1.0	1.4	2.0	5.7	10.3	17.1	16.5	11.8	10.8	8.5	4.1	2.7	2.0	1.7	2.2

[a]Results in this table originate from four calves, aged 16 ± 5 days. Lymph was obtained approximately 10 h after feeding and according to techniques described in the Materials and Methods section.
[b]These fractions were obtained by density gradient ultracentrifugation of intestinal lymph from which chylomicrons and VLDL had been previously removed.
[c]Chylomicrons (S_f > 400) and VLDL (S_f < 400, d < 1.006 g/ml) were separated from each other by sequential ultracentrifugation as described in the Materials and Methods section.
[d]Means of duplicate analyses of lymph fractions from four calves.
[f]ND, not determined, owing to the presence of insufficient amounts of lipoproteins for chemical analysis.
[j]Means ± SD.

should be noted that IDL (defined as lipoproteins with d 1.006–1.026 g/ml) were consistently undetectable.

Examination of our results on the lipid and protein content of calf lymph lipoproteins revealed a number of clearly defined, density-related compositional changes (Table 2). First, the respective chemical compositions of chylomicrons and VLDL, expressed as percents of total lipoprotein mass, were notably different in several respects. Indeed, these two types of particles exhibited mean protein contents of 2.0% and 8.2%, respectively, while the proportion of triglyceride was approximately 10% higher in the former as compared to the latter lipoprotein species. A supplementary difference concerned the ratio % cholesteryl esters/% free cholesterol, whose mean value was 0.82 in chylomicrons but considerably higher (2.17) in VLDL.

Evaluation of the distribution of cholesterol in particles with d > 1.026 g/ml showed that the proportion of this lipid was maximal in the higher density part of the LDL region and over most of the HDL_L density range. Indeed, the sum of the proportions of free cholesterol and cholesteryl ester throughout the d 1.046–1.086 g/ml interval was consistently greater than 30% of the total lipoprotein mass in each subfraction, with a maximum of 35.3% in that of d 1.053–1.060 g/ml. In this region of the density spectrum of calf lymph lipoproteins, the greatest proportion of cholesterol was present in the esterified form, with the ratio % cholesteryl esters/% free cholesterol attaining values as high as 20:1 or 22:1 in lipoproteins with d 1.068–1.091 g/ml. By contrast, this same ratio decreased markedly in HDL subfractions with successively higher densities, attaining a minimum of 0.14:1 in HDL_H of d 1.167–1.180 g/ml.

The density distribution of triglycerides in lipoproteins with d > 1.026 g/ml could be divided into three parts, i.e., 1) from d 1.026 to 1.053 g/ml, in which this lipid accounted for a major proportion of lipoprotein mass, (from 35 to 18%), decreasing with increasing density: 2) from 1.053 to 1.143 g/ml, an interval in which the contribution of triglyceride was both minimal and relatively stable (approximately 4 to 6%); and 3) in denser HDL_H (d > 1.143 g/ml) in which a slight but consistent increase in the proportion of triglyceride (to approximately 8% of lipoprotein mass) was noted.

The highest proportion of phospholipid (32.5%) occurred concomitantly with that of cholesterol in particles of the d 1.053–1.060 g/ml subfractions. Elevated contents of phospholipid (>27%) were also a feature of lipoproteins present in the d 1.053–1.108 g/ml interval; this same type of lipid constituted 20–25% of lipoproteins with lower (d < 1.053 g/ml) and higher (d > 1.108 g/ml) densities, with the exception of denser HDL_H (d 1.154–1.180 g/ml) in which phospholipids decreased progressively to approximately 10% of the total weight of the particles.

Electrophoresis of native lipoproteins

The lipoprotein content of different fractions isolated from calf intestinal lymph was assessed in polyacrylamide gel slabs. Thus (**Fig. 1**), in this system, chylomicrons remained at the origin, while VLDL migrated to the same position as their counterparts in human plasma. Lipoproteins in the d 1.053–1.119 g/ml interval (representing more than 80% of total lipoproteins with d > 1.006 g/ml, see Table 2), presented several populations. Indeed, the migration of the most prominent component approximated that of human plasma HDL, while two faint supplementary bands, both migrating slightly farther into the gel than human LDL, were consistently observed.

Determination of the molecular sizes of lipoprotein particles and of the degree of heterogeneity of density gradient subfractions

Given the extensive range of lipoprotein particle sizes in lymph (ca. 90 to 2400 Å), we applied two methodological approaches for determination of precise diameter. Negative stain electron microscopy was used for size estimation of triglyceride-rich particles (overall size range ≈ 300 to 2400 Å), while gradient gel electrophoretic studies provided data on particles in the size range of ≈ 90 to 215 Å. This choice was based on the inability of electrophoretic methods to permit accurate estimation of the size of particles with diameters greater than ≈ 350 Å. Conversely, determination of the size of HDL with diameters less than ≈ 100 Å by electron microscopy is fraught with error due to difficulties in defining the precise contours of these particles.

Electron microscopic studies. By negative stain electron microscopy, the morphology of both fractions of triglyceride-rich particles resembled each other closely, i.e., essentially spherical particles that deformed readily upon contact. Thus, in a representative preparation of chylomicrons, the overall range of diameters was 650–2400 Å, with a mean ± SD and mode of 1165 ± 352 and 1050 Å respectively (**Fig. 2A**). The distribution of VLDL particle size was distinct from that of chylomicrons, with an overall range of 340–860 Å, a mean ± SD of 570 ± 131 Å and a mode of 550 Å (Fig. 2B).

Gradient gel electrophoresis. This procedure was used to determine the Stokes diameters of lipoproteins with d > 1.006 g/ml, using aliquots from those fractions of density gradients in which sufficient lipoprotein material was present. This type of analysis was performed on the content of all subfractions in which enough lipoprotein material was present, i.e., subfractions 12 to 17 or 11 to 17, according to the lymph sample considered. Results (**Fig. 3**) showed that the material constituting these lipoprotein fractions was uniformly and consistently dominated by particles presenting as a massive band, and whose Stokes diameters decreased with increasing density from 140 to 130 Å in fraction 11 to 115–105 Å in fractions 16 and 17.
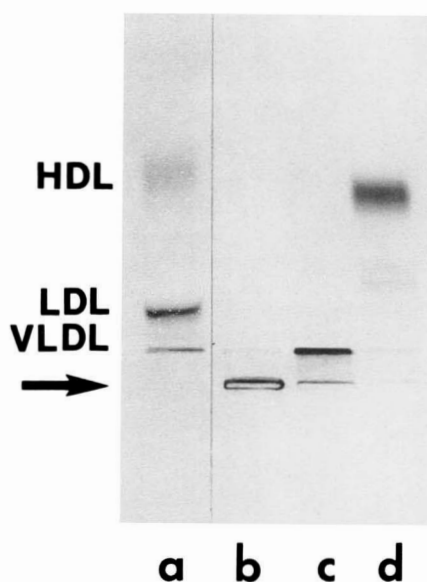

Fig. 1. Polyacrylamide slab gel electrophoresis. Arrow indicates the position of layers. Prestaining was performed using Sudan Black. From left to right: lane a: normal human plasma, with VLDL, LDL, and HDL indicating the respective positions of migration of these different lipoproteins; lanes b, c, and d: lipoproteins from calf postprandial intestinal lymph; lane b: chylomicrons; lane c: VLDL; lane d: total lipoprotein content of the d 1.053–1.119 g/ml interval.

In these two latter fractions, a supplementary and smaller component, exhibiting a diameter in the 95–90 Å range, was also consistently observed.

In addition, fractions 11 to 13 contained small amounts of particles of much larger size, whose quantitative importance decreased with increasing density. These supplementary lipoproteins consisted essentially of a doublet of bands with apparent Stokes diameters of 204–196 Å, followed by a faint band representative of a component with diameter ≈ 215 Å. Finally, a trace component with smaller size (≈ 175 Å) was detected in fraction 11.

Analysis of protein moieties

Apolipoprotein analysis was performed using SDS-polyacrylamide gels of 10% and of 3% monomer concentrations (**Fig. 4**). The pattern of lower M_r proteins in chylomicrons (Fig. 4, gel a) was dominated by a component with M_r ≈ 10,000. Small amounts of proteins with M_r ≈ 27,000 and ≈ 51,000 were also present, together with a trace component of smaller size (M_r 8,800). At the top of the gel, a band representative of an apolipoprotein with M_r > 150,000 was noted. Examination of this same material in gels of low monomer concentration (Fig. 4, gel e) allowed more precise determination of the M_r of the latter protein (320,000). The apolipoprotein content of lymph VLDL was both qualitatively and semi-quantitatively similar to that of chylomicrons, with the exception of a supplementary protein with M_r ≈ 61,000, which was consistently observed (gels b and f).
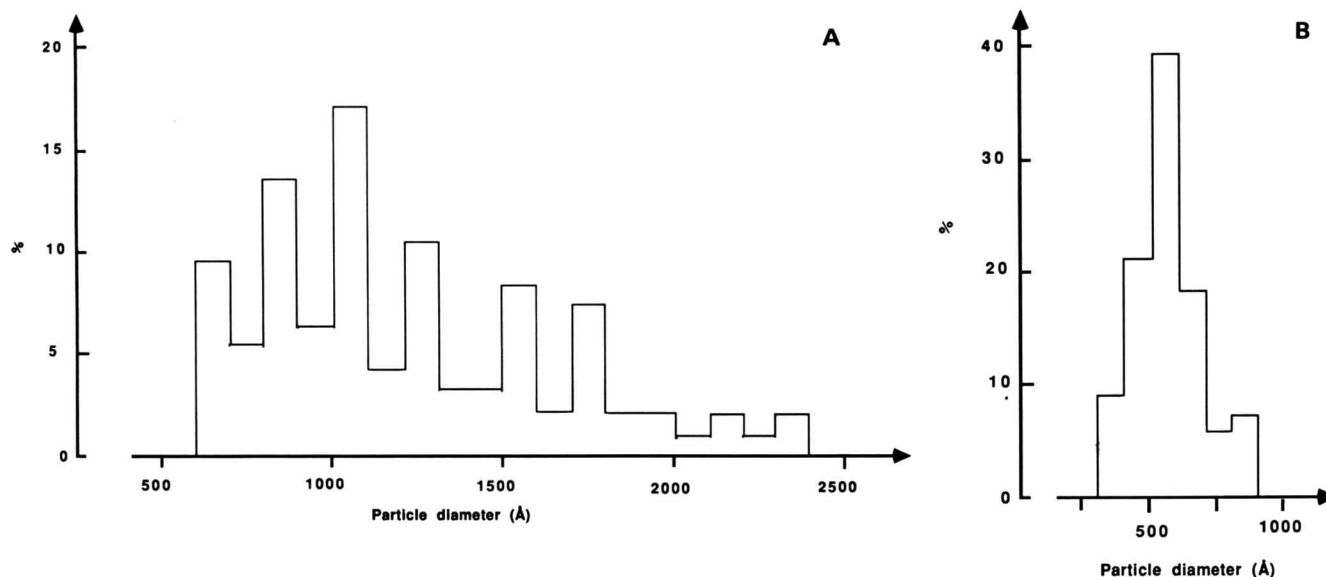
Fig. 2. Frequency distribution of particle diameters in (A) chylomicrons ($S_f > 400$) and (B) VLDL ($S_f < 400$, $d < 1.006$ g/ml) from the intestinal lymph of the preruminant calf. Particles in representative preparations of each fraction were examined by negative stain electron microscopy and diameters were determined as described in the Methods section. The distributions exclude up to 3 % of the occasional particles present at either extreme of the ranges.

Gradient subfractions corresponding to lipoproteins of higher densities (1.053–1.119 g/ml) were also analyzed. Their low M_r ($< 100,000$) protein pattern (gels c and d) was largely dominated by a component with M_r 27,000–28,000, while a peptide with M_r 13,000–14,000 was also present. In addition, small amounts of a protein(s) with M_r in excess of 150,000 were observed in fractions 11 to 13 (d 1.053–1.076 g/ml) at the top of 10 % monomer gels. When proteins from these latter fractions were analyzed in 3 % SDS gels (Fig. 4, gel g), their content of high M_r components was shown to consist of two different proteins with respective M_r values of $\approx 320,000$ and $\approx 580,000$, among which the smaller predominated to a varying degree according to the particular animal. It is of note that a trace of the protein with $M_r \approx 320,000$ was, on occasion, observed in higher density subfractions (up to d 1.108 g/ml, see Fig. 4, gel h).

DISCUSSION

In our preceding report on plasma lipoproteins in the preruminant calf, we presented the chemical and physicochemical characterization of these particles in fasting animals, together with an estimation of lipoprotein fluxes through the liver (7). We now describe the characterization of the lipoproteins of intestinal lymph obtained in the postprandial state at peak lipid absorption, i.e., 10 h after the last meal.

As anticipated, triglyceride was the dominant lipid in our samples. This finding is consistent with the distribution of lipoproteins in intestinal lymph, which consisted

essentially of triglyceride-rich particles and among which chylomicrons were about fourfold more abundant than VLDL. To our knowledge, no other data on the chemical composition of lymph chylomicrons in the bovine are presently available. However, the physical and chemical properties of chylomicrons from our lymph samples compared well with those reported in the literature for lymph chylomicrons in humans and other species (23, 24). In the dairy cow, Ferreri and Elbein (25) examined the composi-
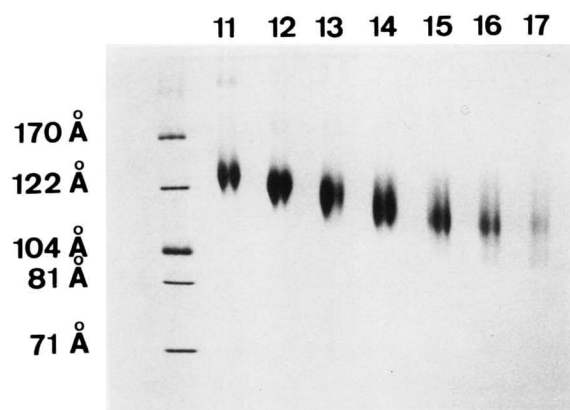

Fig. 3. Polyacrylamide gradient gel electrophoresis of the lipoprotein content of density gradient subfractions from calf postprandial intestinal lymph. The gel slabs contained a 4–30 % monomer gradient (from top to bottom). The number of each subfraction is indicated at the top of the figure (see Table 2 for corresponding densities). A set of standard marker proteins was loaded in the first lane on the left; the standards and their Stokes' diameters were, respectively: thyroglobulin, 170 Å; ferritin, 122 Å; catalase, 104 Å; lactic dehydrogenase, 81 Å; bovine serum albumin, 71 Å. For lipoprotein fractions, 15 μg protein was layered in each lane. Gels were stained with Coomassie Brilliant blue R-250.
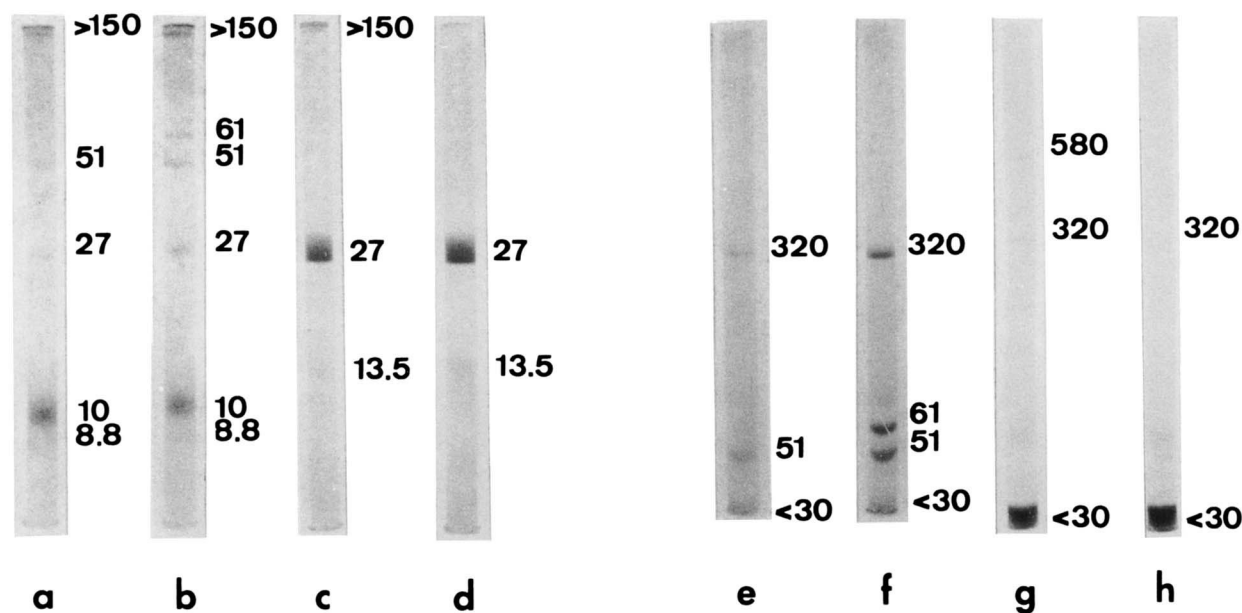
Fig. 4. SDS-polyacrylamide gel electrophoresis of the apolipoprotein moiety of lipoproteins from calf postprandial lymph. From (a) to (d): 10% monomer gels; (e) to (h): 3% monomer gels. Gels (a) and (e): chylomicron apolipoproteins; gels (b) and (f): VLDL apolipoproteins; gels (c) and (g): apolipoproteins from lipoproteins contained in fraction 12 from the density gradient (d 1.060–1.068 g/ml); gels (d) and (h): apolipoproteins in fraction 16 from the density gradient (d 1.091–1.108 g/ml). Molecular weights are expressed in kDa; gels were stained with Coomassie Brilliant blue R-250.

tion of chromatographically separated plasma chylomicrons. Their results revealed a higher proportion of free cholesterol and approximately 50% less phospholipid content as compared to our findings in the calf (Table 2). Such differences are, however, compatible with available information on the consequences of exposure of lymph chylomicrons to serum, i.e., gain of free cholesterol and loss of phospholipid which is recovered in HDL particles (26, 27).

The principal high molecular weight apolipoprotein of lymph chylomicrons was a component with an apparent M_r of $\approx 320,000$ in 3% monomer SDS gels. We have previously documented the presence of a protein with comparable M_r ($\approx 265,000$) in calf plasma lipoproteins with d < 1.076 g/ml (7), and had suggested that this protein could be a counterpart to human intestinal apoB-48 (28). This suggestion was further supported by the fact that the $M_r \approx 265,000$ band appeared maximally in fractions from portal vein plasma (7).

The non-apoB-like apolipoproteins present in lymph chylomicrons primarily consisted of a low M_r ($\approx 10,000$) component together with a minor peptide of M_r 8,800; these polypeptides probably represent members of the C apolipoprotein family. Such proteins could be similar to certain of the $M_r \approx 9,000$–15,000 components present in calf plasma lipoproteins that were detected in the continuous gradient gel system in our previous report (7). We cannot, however, exclude the possibility that the M_r 8,800 polypeptide may correspond to a monomeric form of apoA-II; indeed, Lim and Scanu (29) and Patterson and

Jonas (30) have already suggested that such an apolipoprotein may be a component of bovine HDL.

In addition, two other minor proteins were identified in lymph chylomicrons; we observed counterparts to each of these in calf plasma VLDL (7), with M_r 54,000 (52,000 in lymph) and 28,000, respectively. With regard to the former component, it is of note that β2-glycoprotein-I, originally discovered as a constituent of human serum (31), was shown by Polz and Kostner (32) to be present in all major lipoprotein classes, including postprandial chylomicrons. Subsequently, β2-glycoprotein-I from triglyceride-rich serum lipoproteins was found to be polymorphic, presenting as three isoforms with a common M_r of 54,000 (33).

The M_r 28,000 protein was apparently calf apoA-I, a well-established component of chylomicrons from both lymph and plasma in other species. The proportion of apoA-I in the protein moiety of lymph chylomicrons may vary considerably according to the study considered, ranging from 38 to 50% (27, 34, 35) to as little as 15% (24). The appearance of our SDS-gels suggested that the proportion of apoA-I in calf lymph chylomicrons could be closer to the latter value (Fig. 4, gels a and e).

Particles isolated as lymph VLDL ($S_f < 400$; d < 1.006 g/ml) were similarly enriched in triglycerides, but were distinguished from chylomicrons in that the ratio % cholesteryl esters/% free cholesterol was significantly higher in the former. Thus, the mean ratio in lymph VLDL was 2.17, a value that compared well with values obtained previously for calf plasma VLDL (d < 1.018

g/ml), i.e., 1.78–2.00, according to the site of blood sampling (7). The only noticeable difference between the respective apolipoprotein contents of lymph chylomicrons and VLDL concerned the specific presence, in these latter particles, of a protein with M_r 61,000 that was not previously detected in calf plasma VLDL (7). It is relevant that Fidge and McCullagh (36) have reported the presence of an apoprotein with $M_r \approx 59,000$ in rat lymph VLDL isolated after repeated ultracentrifugation and proposed to term this protein apoA-V; they noted that it displayed physicochemical and lipid-binding properties resembling those of β2-glycoprotein-I.

Finally, it is noteworthy that analysis of the apoprotein content of calf plasma VLDL (7) revealed the presence of an $M_r \approx 42,000$ polypeptide that was specifically associated with the triglyceride-rich lipoproteins of calf plasma (d < 1.024 g/ml), and with the denser HDL_H subclass in which the proportion of triglyceride was increased as compared to HDL particles of lower density. On the basis of these criteria, we suggested that this protein be termed bovine apoA-IV. ApoA-IV (M_r in humans, $\approx 46,000$ (37)), is a component of newly secreted chylomicrons and VLDL in humans and the rat (38), in which it accounts for approximately 10% of the protein moiety (24, 38). When such particles enter the blood circulation, this apoprotein rapidly transfers predominantly to HDL in the rat (39). By contrast, contradictory results have been reported in humans, suggesting that apoA-IV either associates preferentially with HDL (40) or is primarily present in free form sedimenting in the d > 1.21 g/ml infranate upon centrifugation (41). It is therefore surprising that no protein akin to apoA-IV in size could be detected in our preparations of triglyceride-rich lymph lipoproteins. Apart from the possible absence of authentic apoA-IV in bovine species, possible explanations for this finding could include: 1) the presence of a very low proportion of apoA-IV in the protein moiety of both chylomicrons and VLDL, making this protein undetectable in our gels, and 2) a higher M_r for bovine apoA-IV as compared to its human counterpart; in this case, the component that we consistently detected with M_r 51,000 may correspond to this protein.

The fact that the M_r 61,000 apoprotein was consistently observed in preparations of lymph VLDL but not in chylomicrons suggests that some difference may exist between the two lipoprotein fractions, either in the process of their biosynthesis in the intestinal cell, or in their ability to exchange or accept components from other lymph lipoproteins. Indeed, evidence from analysis of the respective contents of apoB in chylomicrons and VLDL from human chylous ascitic fluid (42), and from the limited mixing of chylomicrons and VLDL-size particles in secretory vesicles from isolated rat epithelial cells (43), suggests that these two lipoprotein fractions contain distinct particle species and that their biosynthesis is subject

to subcellular compartmentalization. It should be pointed out that all preparative ultracentrifugations involved in the purification of our chylomicron and VLDL fractions were performed at 15°C, enabling us to avoid crystallization of core triglyceride (especially saturated triglyceride) which leads to the appearance of artifactual "VLDL" or of unusual intermediate density lipoproteins in the cow (44).

In lymph samples obtained 10 h after the meal, i.e., at peak lipid absorption, the mean value of the ratio of the concentration of chylomicrons to VLDL was approximately 4:1 (range 3.2 to 5.2:1, according to the animal considered). It is, however, well established that the particle size of chylomicrons is modified according to the lipid flux transported (45), and therefore that particular chylomicron size increases during maximal lipid absorption. Thus, it could be expected that when lower lipid fluxes are accommodated, i.e., at the beginning and at the end of digestion, the concentration ratio of chylomicrons to VLDL is modified, to the benefit of the latter particles.

Apart from triglyceride-rich particles with hydrated densities less than 1.006 g/ml, the major components of the lipoprotein spectrum in our lymph samples were those distributed over the 1.053–1.119 g/ml density range. Electrophoretic analyses in SDS-polyacrylamide gels showed that the M_r 28,000 protein predominated in every subfraction in this density interval. As in the case of chylomicrons and VLDL, we suggest that this polypeptide is calf apoA-I. Indeed, we have recently isolated a protein with similar M_r from d 1.060–1.180 g/ml calf plasma lipoproteins, and characterized it as authentic apoA-I (46). The presence of apoA-I-rich HDL-like particles in lymph gradient subfractions with d 1.053–1.119 g/ml was further confirmed by gradient gel electrophoresis of native lipoproteins. These studies permitted identification of a spectrum of particles whose Stokes diameters decreased with increasing density and resembled human HDL of corresponding hydrated density (17). In contrast, the lipoprotein content of the d 1.053–1.119 g/ml interval of postprandial calf intestinal lymph was clearly heterogeneous, at least with respect to the gradient subfractions of lower density (< 1.076 g/ml). Indeed, two high M_r apolipoproteins, whose sizes were comparable to those of human apoB-100 and apoB-48, were present in such fractions. In line with this finding, at least two quantitatively minor lipoprotein populations with Stokes diameters corresponding to those of denser LDL (17) were identified in these same subfractions by gradient gel electrophoresis. The presence of a trace component with diameter ≈ 175 Å was also noted. Such particle heterogeneity renders interpretation of our chemical analyses of such gradient subfractions difficult. However, the subfractions displaying the highest proportions of cholesteryl ester (> 30% of lipoprotein mass) were distributed over the density interval from 1.053 to 1.076 g/ml, i.e., subfractions in which

particles with Stokes diameters typical of LDL and apolipoproteins with apoB-like molecular weights were present. The application of preparative methods based on the distinct apolipoprotein content of LDL- and HDL-like particles should permit separation of the different types of lipoproteins contained in the 1.053–1.076 g/ml density range (47–49).

It is generally considered that the small amounts of mesenteric lymph LDL observed in other species (e.g., the rat) represent filtered plasma LDL (24, 38). As regards the calf, our previous study (7) has provided evidence for the occurrence of the two major forms of apoB in all density gradient fractions of plasma lipoproteins up to a density of 1.076 g/ml. On the basis of their apolipoprotein content, it is therefore conceivable that the LDL-like particles in intestinal lymph may originate from equivalent lipoproteins filtered from the plasma compartment. However, the respective intensities of the bands corresponding to the two forms of apoB-like proteins varied according to the sample considered, the lower M_r form predominating in all cases. These findings are consistent with a process in which both intestinal secretion of particles and filtration from plasma would be involved, with each of these physiological phenomena proceeding at its own rate and differing from one animal to another.

Very small amounts of lipoproteins with d > 1.119 g/ml (subfractions 18–22; Table 2) were present in our lymph samples and therefore only compositional data could be obtained. These data disclosed two features of potential importance, however. First, and in contrast to plasma particles occurring within a similar density range (7), the ratio % cholesteryl ester/% free cholesterol decreased with density, from 7.0:1 in the d 1.119–1.131 g/ml subfraction to 0.14:1 in the highest density subfraction (1.167–1.180 g/ml). In the corresponding density gradient subfractions from plasma, such values were considerably higher (approximately 13:1 and 15:1, irrespective of the site of sampling (7)). To account for the elevated content of cholesteryl esters in HDL_H from calf plasma, we had suggested two complementary mechanisms. The first involved the low activity of cholesteryl ester transfer protein (CETP) in bovine species (50). In addition, the presence in plasma, at least in the fasting state, of low concentrations of lipoproteins that could potentially act as cholesteryl ester acceptors (i.e., VLDL and LDL) could also be responsible for this compositional feature. By contrast, the presence in lymph of large amounts of both chylomicrons and VLDL could provide suitable acceptors for esterified cholesterol. Furthermore, the observation that the apparent mass of CETP in human plasma increases rapidly in response to an increase in dietary fat (51) has led Tall (52) to suggest that CETP synthesis could occur in cells directly involved in lipid flux, such as enterocytes. It is also relevant here that lipoprotein lipase enhances the CETP-mediated transfer of cholesteryl esters from HDL

to triglyceride-rich lipoproteins in vitro (53). Thus, our observations on the lipid composition of lymph HDL in the calf are consistent with those of Green, Tall, and Glickman (54), who reported that the entire HDL fraction in rat mesenteric lymph was poor in cholesteryl ester and enriched in phospholipids.

A second compositional feature distinguished the calf lymph lipoproteins of d > 1.119 g/ml and, contrary to that described above, was shared with the corresponding fractions in plasma (7). Indeed, in both lymph and plasma, HDL subfractions of highest density displayed an increase in the proportion of triglycerides with increase in density, from approximately 4% in the d ≈ 1.100 g/ml region in lymph (≈ 1% in corresponding plasma lipoproteins) to more than 8% in the 1.167–1.180 g/ml subfraction (plasma: 5–6%). In calf plasma, the slight increase in the proportion of triglyceride in the HDL_H subfractions with highest density was accompanied by the appearance of an apoprotein with M_r ≈ 42,000 which we tentatively identified as apoA-IV. If such a protein is actually present in postprandial lymph (see above), then a similar phenomenon could occur. Unfortunately, due to the minute amounts of material present in the highest density subfractions of lymph gradients, we were unable to perform electrophoretic analyses on their lipoprotein and apoprotein content. Nonetheless, we did detect a supplementary population of HDL-like particles, with Stokes diameters of approximately 95–90 Å, in the density fractions submitted to gradient gel electrophoresis (i.e., those with d 1.091–1.108 g/ml and 1.108–1.119 g/ml). These lipoproteins could represent the lowest density part of the distribution of small, apoA-IV-containing, HDL particles, and as such would correspond to those exhibiting diameters of 78–80 Å and described by Bisgaier et al. (55) in normal human plasma.

The present study was performed in the preruminant calf, an animal whose size and anatomical characteristics readily allow physiological investigations of hepatic and intestinal function and the withdrawal of large volumes of blood and mesenteric lymph. We have presently established that the calf possesses several distinct species of lipoprotein particles in this mesenteric lymph that are comparable to those observed in the rat and in humans. Taken together, these arguments suggest that the calf could be a suitable and important animal model for kinetic and metabolic studies on intestinal lipid and lipoprotein metabolism. ▊

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