Intestinal lymph and plasma lipoproteins in the preruminant calf: partial resolution of particle heterogeneity in the 1.040–1.090 g/ml interval

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Abstract Our previous studies in the preruminant calf have provided evidence for the heterogeneity of lipoprotein particles in the 1.040–1.090 g/ml density interval in both plasma and postprandial intestinal lymph (Bauchart, D. et al., 1989. *J. Lipid Res.* **30:** 1499–1514; and Laplaud, P. M. et al., 1990. *J. Lipid Res.* **31:** 1781–1792). We therefore attempted to resolve this heterogeneity by use of heparin-Sepharose affinity chromatography. Experiments were performed on three calves; portal vein plasma and intestinal lymph were obtained simultaneously 10 h after a meal, i.e., at peak lipid absorption. In both fluids, the chromatographic profile presented three fractions, I, II, and III. Fraction I was characterized by the presence of cholesteryl ester-rich particles (≈35–37% of lipoprotein mass), which migrated electrophoretically as typical high density lipoproteins and exhibited Stokes diameters in the 130–160 Å range; apoA-I was the predominant protein. In addition to this polypeptide, fraction II contained small amounts of a supplementary protein ($M_r \approx 51{,}000$), exhibiting heparin-binding properties. In the light of results reported in the literature, we suggest that this latter protein could correspond to β_2 glycoprotein I. The chemical composition of each fraction II closely resembled that of the corresponding fraction I, while their electrophoretic migrations appeared slightly slower and their Stokes diameters slightly larger (155–165 Å). Apart from the presence of small amounts of apoA-I, two high M_r proteins (M_r approx. 560,000 and 300,000) were typical of the apolipoprotein moiety of fractions III. The lower M_r form was present as a trace component only in fraction III originating from plasma; its proportion increased in lymph fraction III so as to approximately match that of the higher M_r (i.e., 560,000) protein. In both plasma and lymph, fraction III was electrophoretically heterogeneous, exhibiting a doublet of bands with migration and Stokes diameters (250 Å) typical of low density lipoprotein particles. However, no evidence for the presence of a particle resembling lipoprotein[a] in fraction III could be obtained. In lymph only, fraction III contained a supplementary population of lipoproteins with migration intermediary between those of conventional low and high density lipoproteins and with Stokes diameters in the 190–200 Å range. Other specific features of lymph fraction III included a sevenfold increase in its triglyceride content (8.5 ± 3.4% vs. 1.2 ± 1.1% in the corresponding fraction from plasma), to the detriment of cholesteryl esters, and a higher proportion of protein. These results provide the basis for future experiments regarding the metabolism of the different populations of lipoprotein particles which we have presently characterized.—**Laplaud, P. M., D. Bauchart, D. Durand, L. Beaubatie, and M. J. Chapman.** Intestinal lymph and plasma lipoproteins in the preruminant calf: partial resolution of particle heterogeneity in the 1.040–1.090 g/ml interval. *J. Lipid Res.* 1991. **32:** 1429–1439.

Supplementary key words intestinal lymph duct • portal vein • LDL • cannulation in vivo • cholesteryl ester-rich HDL • heparin affinity chromatography • apolipoproteins

Recent collaborative studies from our group have established the preruminant calf as an important animal model for studies on the respective roles of the liver (1) and the intestine (2) in lipoprotein metabolism. Advantages of this temporarily monogastric animal include easy access to hepatic and intestinal blood vessels and possible withdrawal of large volumes of both blood and intestinal lymph without undue stress to the animal.

In our original report (1), we described the characterization of lipoproteins present in the afferent (portal vein and hepatic artery) and efferent (hepatic vein) hepatic vessels in the fasting calf. Subsequently, we analyzed the lipoproteins and apolipoproteins present in calf intestinal lymph at peak lipid absorption, i.e., ca. 10 h after a meal (2). In both cases, we demonstrated that a specific region of the lipoprotein density distribution, approximately corresponding to the 1.040–1.090 g/ml density interval, contained a complex mixture of lipoproteins. Indeed, distinct particles with either HDL-or LDL-like electrophoretic properties were simultaneously detectable over this den-

Abbreviations: EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, apolipoprotein.

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sity range. In addition, the latter lipoproteins appeared heterogeneous in intestinal lymph (2).

Clearly then, subfractionation of plasma and lymph lipoproteins distributed within the density interval of 1.040–1.090 g/ml was required in order to characterize the individual particle species. We have addressed this question by use of heparin-Sepharose affinity chromatography, a method that we have used earlier to solve a similar separation problem encountered in the European badger (3). We presently describe our data in the calf using this methodology; blood and lymph were drawn in a postprandial state in order to facilitate comparison of results. In order to compare the lipoprotein content of intestinal lymph with that of plasma emanating from the intestine, we chose to study portal vein plasma. A preliminary report on some aspects of the present studies was presented at the International Conference on Intestinal Lipid and Lipoprotein Metabolism in 1988 (4).

MATERIALS AND METHODS

Animals and diets

Three crossbred Friesian-Holstein male calves were used in our experiments. These animals were provided by the Laboratory of Research on Lactation (INRA, Theix, France) and were housed individually in wooden stalls on a litter of wood shavings in an air-conditioned room (average temperature: 20°C, hygrometric level: 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%) acids.

At the time of operation and sampling of biological fluids, the calves were 23 ± 2 days old (mean ± SD), their body weights were 48 ± 5 kg, and they exhibited a growth rate of 483 ± 122 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 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 (5), who demonstrated that maximum lipid absorption in the calf occurred approximately 10 h after feeding.

Surgical techniques

Animals were equipped with a portal vein catheter (polyvinyl 1.5 mm ID × 2.5 mm OD) as previously described (1). Another catheter was introduced into the main intestinal lymph duct according to the technique of Romsos and McGilliard (6). 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.

Blood and lymph samples

Thirty ml of blood was collected from the portal vein in Na_2-EDTA (final concentration 1 mM). Plasma was then separated by centrifugation at 3500 rpm for 15 min and antibacterial agents were added (sodium merthiolate and sodium azide at final concentrations of 0.001% and 0.01% (w/v), respectively). Plasma was maintained at 4°C until lipoprotein fractionation was initiated and typically within 48 h of its isolation (see "Lipoprotein isolation" below). Approximately 200 ml of lymph was obtained from the main intestinal duct while the animal was under halothane anesthesia (1). Sodium-EDTA and sodium azide at the same concentrations as above were immediately added and lymph was stored at 4°C until lipoprotein fractionation began, i.e., usually within 24 to 48 h of surgery.

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

The enzymatic techniques used for measurement of the concentrations of the different classes of lipids, both in total plasma and lymph, and in lipoprotein fractions, have been described elsewhere (7). The method of Lowry et al. (8) was used for the assay of protein concentrations using bovine serum albumin as standard.

Lipoprotein isolation

Sequential isolation of d 1.040–1.090 g/ml lipoproteins from both plasma and lymph was performed using established procedures (9), and adapted to the MSE 8 × 14 ml aluminum fixed-angle rotor. All centrifugations reported in this paper were carried out in MSE Prepspin 50 ultracentrifuges (MSE Scientific Instruments, Crawley, UK). After ultracentrifugation, lipoprotein fractions were dialyzed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA, exclusion limit 6,000–8,000) for 3 × 12 h at 4°C against a solution containing 0.15 M NaCl, EDTA (1 mM), and sodium azide (0.02%), and concentrated to the desired volume using an Amicon 8 MC micro-ultrafiltration system fitted with XM50 ultrafiltration membranes, exclusion limit 50,000 (Amicon, Lexington, MA).

Affinity chromatography

The present experiments were based on our modification (3) of the methodology reported by Weisgraber and

Mahley (10) for subfractionation of human HDL. Thus, the concentrate of d 1.040–1.090 g/ml lipoproteins (15–20 mg of lipoprotein protein) in 2–3 ml of NaCl-Tris buffer (0.115 M NaCl, 0.005 M Tris, pH 7.4) to which $MnCl_2$ (final concentration 0.025 M) was added immediately before application of the sample to the column, was subjected to affinity chromatography on a 12 × 300 mm glass column (LKB, Bromma, Sweden), operated at 4°C. The column had been filled with heparin-Sepharose prepared from heparin (Hynson, Westcott, Dunning, Inc., Baltimore, MD) and Sepharose CL 6B (Pharmacia Fine Chemicals), according to Weisgraber and Mahley (10).

Elution with the manganese-containing buffer (application buffer described above) was continued until lipoprotein fraction I (see Results) had been collected. At this point, the manganese was removed but the NaCl concentration in the eluting buffer remained unchanged; this modification resulted in the elution of lipoprotein fraction II. After the elution of this latter fraction was complete, the NaCl concentration in the buffer applied to the top of the column was increased to 0.29 M, thereby effecting elution of lipoprotein fraction III. A final increase in the NaCl concentration of the eluant to 0.6 M was then made.

The column effluent was monitored at 280 nm using an LKB Uvicord detector and recorder. After collection of successive fractions, those corresponding to each peak were pooled, concentrated using the same apparatus described above, and dialyzed against three changes of a 0.15 M NaCl solution for 36 h at 4°C.

Electrophoretic methods

Non-denaturing polyacrylamide gel electrophoresis of plasma lipoproteins and of lipoprotein fractions was performed using commercially available polyacrylamide gel slabs (Lipofilm, Sebia, Issy les Moulineaux, France). These slabs contained a discontinuous acrylamide gradient from 2% (at point of sample application) to 3% (running gel). No attempt was made to quantify the various components observed.

Non-denaturing continuous-gradient slab-gel electrophoresis was performed on a Pharmacia electrophoresis apparatus GE 2/4 loaded with PAA 2/16 gradient gels (Pharmacia Fine Chemicals), according to conditions described earlier (11). The Stokes diameters of the particles were calculated using the Stokes-Einstein equation as described by Anderson et al. (12). Prior to electrophoretic examination of the content of their apolipoprotein moiety, lipoproteins were first delipidated with ethanol–diethyl-ether 3:1 (v/v) as described by Brown, Levy, and Fredrickson (13); the apolipoprotein residue was then dried under N_2. The molecular weights of apolipoproteins were estimated: *1*) by electrophoresis in SDS-polyacrylamide gels of either 10% monomer concentration as described by Weber and Osborn (14), or 3% monomer concentration according to the modification of Weisgraber et al. (15) of the methodology of Stephens (16), and *2*), by electrophoresis on PAA 2/16 gradient gels (see above), using a 25 mM Tris-glycine, 0.1% SDS migration buffer, pH 8.3, and other experimental conditions as specified by the manufacturer. According to the particular experiment considered, calibration curves for estimation of molecular weights were constructed from different series of molecular weight markers, i.e., High and Low Molecular Weight electrophoresis calibration kits from Pharmacia Fine Chemicals, and Electran from BDH Biochemicals (Poole, U.K.). Staining of the gels was performed using either Coomassie Brilliant blue R250 (10% monomer gels) or the technique of Karlson et al. (17) (3% monomer gels and gradient gels).

Double immunodiffusion

Double immunodiffusion was carried out by the technique of Outcherlony (18) in 1% agarose. Aliquots from calf plasma and from the content of affinity chromatography fraction III from both plasma and lymph were reacted with antiserum against human Lp[a] (Immuno AG). Human reference standard to Lp[a] from the same manufacturer was included as control. This experiment was repeated using a variety of protein concentrations (10–50 μg lipoprotein protein) from the calf lipoprotein samples.

RESULTS

Plasma and lymph lipids

Data concerning plasma and lymph lipids are shown in **Table 1.** Results obtained from portal vein plasma in the present experiments are not directly comparable with those already reported by us (1), as, in our earlier experiments, the animals had been fasted overnight. Thus, as expected, our newer data obtained during lipid absorption showed an enrichment in all classes of lipids, whose respective concentrations had approximately doubled with reference to those measured in the fasting state. However, it is of note that the respective proportions of the different major classes of lipids (cholesteryl esters, free cholesterol, triglycerides, and phospholipids) remained very similar between the two series of experiments; indeed, differences in their percent proportions were consistently in the 1–4% range (mean percent proportions of total plasma lipids observed in ref. 1 and in the present study were, respectively: free cholesterol, 5.6 and 4.0%; cholesteryl esters, 42.8 and 47.3%; triglycerides, 10.5 and 9.9%; and phospholipids, 41.0 and 38.7%). Such a consistency in the percent lipid distribution was also observed in the two successive series of data obtained from postprandial intestinal lymph in our previous report (2) and in the present study (mean percent proportions of total

TABLE 1. Total concentrations of the major classes of lipids in portal vein plasma and intestinal lymph at peak lipid absorption

	Lipid Concentration[a]			
	Free Cholesterol	Cholesteryl Esters	Triglycerides	Phospholipids
	mg/dl			
Portal vein plasma	14.2 ± 4.4[b]	166.3 ± 22.7	34.9 ± 10.8	136.2 ± 11.7
Intestinal lymph	29.9 ± 2.1	28.0 ± 5.5	527.5 ± 95.8	104.2 ± 18.7

[a]Each analysis was performed in duplicate on samples from each of three calves.
[b]Values are expressed as means ± SD.

lymph lipids in ref. 2 and in the present study were, respectively: free cholesterol, 1.8 and 4.3%; cholesteryl esters, 3.5 and 4.1%; triglycerides, 80.3 and 76.5%; and phospholipids, 14.4 and 15.1%). However, it is of note that the mean triglyceride concentration measured in lymph in the present experiments was only approximately half that reported in ref. 2.

Affinity chromatography

Elution profiles and determination of the apolipoprotein content of lipoprotein fractions. On a qualitative basis, all plasma and lymph lipoprotein samples studied presented similar elution characteristics from the heparin-Sepharose column, exhibiting three distinct fractions (**Fig. 1**); these components are hereinafter termed fractions I, II, and III.

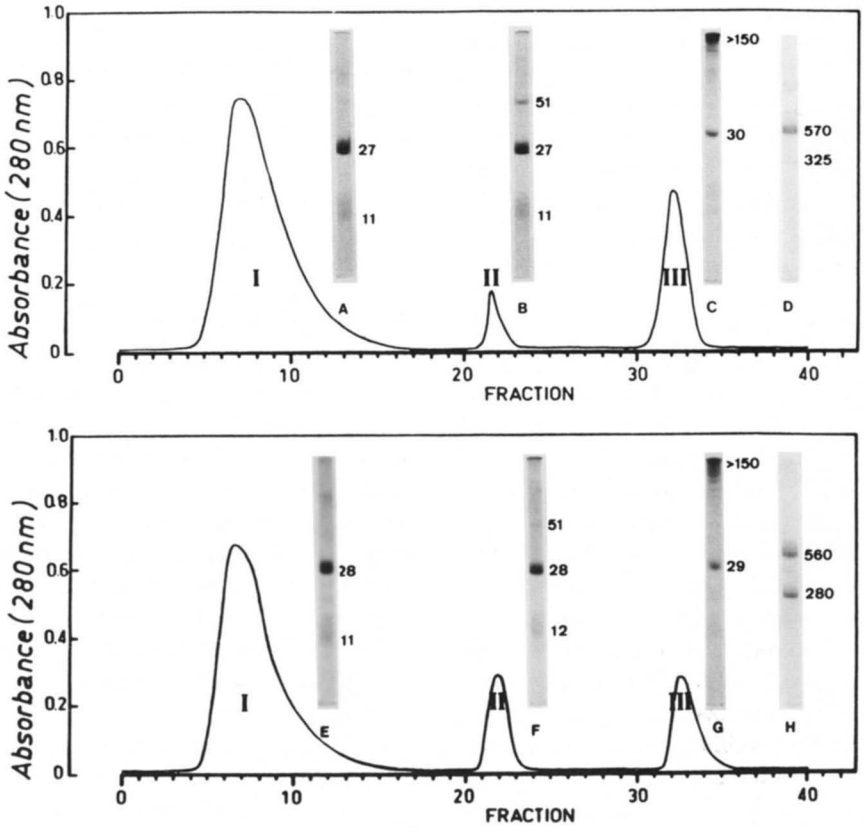

Fig. 1. Subfractionation by heparin-Sepharose affinity chromatography of d 1.040–1.090 g/ml lipoproteins from portal vein plasma (upper diagram) and postprandial intestinal lymph (lower diagram). The elution profiles shown are qualitatively and semi-quantitatively representative of those obtained from samples obtained in the three animals used in the present study (see Results and Table 2). In each case, 15–20 mg of lipoprotein protein in 0.005 M Tris buffer (pH 7.4) containing 0.115 M NaCl and 0.025 M $MnCl_2$, was applied to a 12 × 300 mm column. The column was operated at a flow rate of 24 ml/h and fractions of 4 ml were collected. Gels A, B, and C, and E, F, and G: electrophoresis of the apolipoproteins of fractions I, II, and III from portal vein and intestinal lymph lipoproteins, respectively, in 10% SDS-polyacrylamide gels. Gels D and H: electrophoresis of apolipoproteins of fractions III from portal vein and intestinal lymph, respectively, in 3% SDS-polyacrylamide gels. About 70–100 μg protein was applied to each gel. Molecular masses are expressed in kDa.

Electrophoresis in SDS-polyacrylamide gels of either 10% or 3% monomer concentration provided evidence for the distinct apolipoprotein content of the three chromatographic fractions (Fig. 1). In both plasma and intestinal lymph, fraction I was characterized by the prominent presence of a polypeptide with $M_r \approx 27{,}000$–$28{,}000$ thereby resembling human apoA-I (Fig. 1, gels A and E). It is relevant here, first that fraction I displayed an electrophoretic behavior typical of HDL particles (see below); second, that, in previous studies, we (3) and others (10) have demonstrated that apoA-I-containing lipoprotein particles typically elute in this initial part of the chromatographic gradient, and third, that we have recently purified and physicochemically characterized apoA-I from calf HDL; this protein exhibits an M_r of 27,000–28,000 upon SDS-gel electrophoresis (19). Therefore, it seems reasonable to conclude that the major apolipoprotein of fraction I in the present experiments was actually apoA-I. Other components of the apolipoprotein profile seen in gels of fraction I were limited to a polypeptide with $M_r \approx 11{,}000$; this protein was, however, present in only trace amounts in both plasma and lymph samples. By contrast, the upper section of the 10% gels corresponding to fraction I was consistently free of any trace of staining material which would have been indicative of the presence of high M_r (ca. >150,000) proteins.

Fraction II, which eluted after manganese had been removed from the eluting buffer, had an apolipoprotein profile that was equally dominated by an apoA-I-like protein (Fig. 1, gels B and F). In addition, traces of a polypeptide with M_r 11,000–12,000 were, as in fraction I, equally detectable in lymph samples. Thus, the only noticeable difference between fractions I and II as regards their respective apolipoprotein contents was represented by the consistent presence of small amounts of a protein with M_r 51,000 in fraction II (Fig. 1, gels B and F). Finally, and as observed in fraction I, high M_r component(s) were not detectable at the top of 10% monomer gels of fraction II.

The protein moiety of fraction III from both lymph and plasma was clearly distinct from those of fractions I and II. Indeed, the profile of low M_r proteins was restricted to small amounts of a polypeptide with apparent M_r 28,000–30,000 in fraction III from every plasma or lymph sample examined (Fig. 1, gels C and G). On the contrary, high M_r proteins, whose migration was restricted to the top of 10% monomer gels, predominated; SDS gels of low (3%) monomer concentration were therefore used to allow examination of their distribution. Thus, in such gels, these latter proteins were essentially represented by an apoB-like component with $M_r \approx 570{,}000$ (569,536 ± 16,789, mean ± SD, n = 3) in fraction III from portal vein plasma (Fig. 1, gel D). In addition, a supplementary protein with apparent M_r 323,497 ± 7,987, and thus slightly higher than that originally reported for human apoB-48 (264,000; ref. 20), was consistently detectable as a trace component only. In contrast, fraction III from lymph samples exhibited the presence of two high M_r proteins in approximately equal amounts (Fig. 1, gel H). The M_r of the larger component was comparable to that of its counterpart in plasma (558,655 ± 14,305). However, the smaller protein displayed an apparent M_r (277,638 ± 8,065) lower than the corresponding component observed in trace amounts in fraction III from portal vein samples. This discrepancy might be explained by the well-known observation that the mobility of high M_r proteins, and especially of apoB forms, upon SDS-polyacrylamide electrophoresis, is to some extent dependent upon the amount applied on the gel (20). Therefore, to verify the influence of loading as well as to eliminate possible gel-to-gel variations in electrophoretic mobilities, various amounts of apolipoprotein samples from fractions III originating, respectively, from plasma or lymph obtained from one calf were simultaneously run on gradient gel slabs with 2–16% monomer concentrations. In such gels, protein migration was continued until it virtually stopped after reaching a region of the gel where the average pore size did not allow it to progress further, taking into account its size and shape. Thus, use of this electrophoretic system largely minimized previously observed differences between the respective apparent M_r of the smaller apoB-like protein from plasma and lymph fraction III (see Fig. 2 for an example of gel appearance). Molecular weights were determined using protein loadings calculated such that the respective intensities of staining of the bands corresponding to the smallest apoB-like component were approximately similar for plasma and lymph samples. Under these conditions, the M_r values were indistinguishable from each other (protein contained in fraction III from plasma: 293,512 ± 3,115; from lymph: 290,686 ± 4,113; mean ± SD from three gels; NS).

Gradient slab gel electrophoresis was similarly used to study the influence of nonreducing versus reducing conditions on the distribution of high M_r proteins from fraction III. **Fig. 2** shows that results regarding the bands representative of the two prominent apoB-like proteins were essentially identical under both types of conditions, and irrespective of the plasma or lymph origin of the sample. However, it is of note that faint bands representative of very high M_r components (600,000–1,000,000), and possibly corresponding to aggregated proteins, were apparent in nonreduced samples only.

Finally, calf plasma as well as fractions III from both plasma and lymph were immunologically tested by Outcherlony's method for the possible presence of the [a] antigen, using a polyclonal antibody to human Lp[a]. In each case, no precipitation line could be observed (not shown).

From a quantitative viewpoint, fraction I predominated in the total d 1.040–1.090 g/ml lipoproteins in both

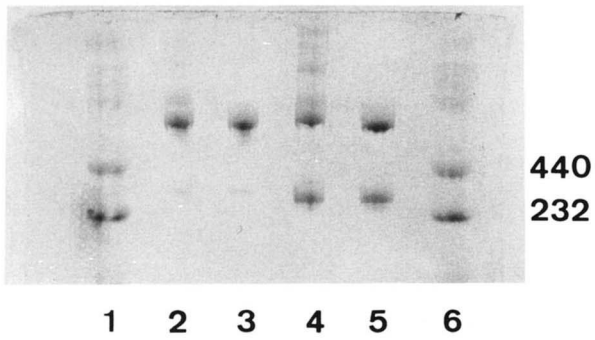

Fig. 2. Gradient gel electrophoresis (2–16% monomer concentration) in 25 mM Tris-glycine, 0.1% SDS migration buffer, pH 8.3, of the high M_r proteins contained in affinity chromatography fraction III. Lanes 1 and 6: protein standards, with M_r (in kDa) indicated on the right of the figure. Lanes 2 and 3: high M_r apolipoproteins from plasma fraction III, unreduced (lane 2), and reduced using 10 mM DTT (lane 3). Lanes 4 and 5: high M_r apolipoproteins from intestinal lymph fraction III unreduced (lane 4), and reduced under similar conditions (lane 5). In each case 10 μg protein was loaded onto the gel. Staining was performed using Coomassie Brilliant blue.

physiological fluids assayed (**Table 2**). Indeed, fraction I accounted for approximately 70% of this material in plasma and for almost 80% in postprandial intestinal lymph. By contrast, fraction II was always representative of a quantitatively minor lipoprotein species (approximately 5% and 10% of total material in portal vein plasma and in intestinal lymph, respectively). Finally, the most significant difference between the two series of samples resided in the twofold increase seen in the relative proportion of fraction III in plasma, as compared to lymph (mean values, ca. 23% vs. 12%). It should be pointed out at this juncture that while SD values pertaining to the respective percentages of the different fractions in total d 1.040–1.090 g/ml lipoproteins were consistently small, those corresponding to the mean concentrations of these same fractions werre considerably greater (Table 2). This finding reflects the fact that the concentrations of total d 1.040–1.090 g/ml lipoproteins were considerably lower in the third calf studied and especially as regards portal vein plasma (animal 1, 160.6 mg/100 ml; animal 2, 192.5 mg/100 ml; animal 3, 51.7 mg/100 ml). In intestinal lymph, however, this phenomenon was less pronounced (33.0, 21.3, and 14.2 mg/100 ml, respectively). However, no noticeable difference between lipoproteins isolated from calf 3 and those originating from the two other animals could be detected by any of our analytical techniques. We have therefore decided to present data obtained in the three calves investigated successively.

The respective chemical compositions of the chromatographic fractions isolated on the one hand from intestinal lymph and on the other from portal vein plasma are presented in **Table 3.** Thus, each series of lipoprotein subfractions isolated from portal vein plasma and from postprandial lymph were cholesterol-rich. Most of this lipid was in esterified form; indeed, the proportion of cholesteryl ester in fractions I and II from both fluids was similar (35–37% of total lipoprotein mass). The percentage of cholesteryl ester in fraction III was even higher, and especially in the plasma lipoproteins ($\approx$48% of particle mass). Phospholipids were the second major lipid constituent of all fractions; the proportion of phospholipid was, however, reduced by approximately one-third in fractions III (ca. 20% of lipoprotein mass), from both fluids as compared to fractions I and II (30% approx., irrespective of the original fluid). Triglyceride was a quantitatively minor component in all series of fractions assayed but one. Furthermore, the respective proportions of this lipid were similar in fractions I, II, and III from portal vein plasma (<1% approx.). However, while fractions I and II from intestinal lymph both contained small amounts of triglyceride (ca. 2%), fraction III from the same fluid exhibited marked enrichment in this lipid (8.6 ± 3.4% of mass, mean ± SD). Finally, the proportion of protein was similar in all chromatographic fractions originating from intestinal lymph (ca. 30% of total lipoprotein mass). In contrast, some differences in protein content were noted between fractions originating from plasma, and especially with regard to fraction III in which protein accounted for 21.1 ± 1.4% as compared to 25.7 ± 1.8% in fraction II and 29.0 ± 4.8% in fraction I.

TABLE 2. Portal vein plasma and postprandial intestinal lymph lipoproteins in the preruminant calf: concentrations of total d 1.040–1.090 g/ml substances and of their respective subfractions isolated by affinity chromatography on heparin-Sepharose

	Portal Vein				Intestinal Lymph			
	Total d 1.040–1.090 g/ml Lipoproteins	Fraction I	Fraction II	Fraction III	Total d 1.040–1.090 g/ml Lipoproteins	Fraction I	Fraction II	Fraction III
Concentration (mg/dl)	134.9 ± 73.8[a]	98.1 ± 56.3	7.5 ± 5.4	29.3 ± 13.0	22.8 ± 9.5	17.9 ± 7.7	2.2 ± 0.9	2.7 ± 0.9
Percent of total d 1.040–1.090 g/ml lipoproteins		71.4 ± 3.2	5.2 ± 1.4	23.3 ± 4.5		78.2 ± 1.2	10.0 ± 1.2	11.8 ± 0.8

[a]Means ± SD (n = 3).

TABLE 3. Portal vein plasma and postprandial intestinal lymph lipoproteins in the preruminant calf. Chemical composition of total d 1.040–1.090 g/ml substances and of their respective subfractions isolated by affinity chromatography on heparin-Sepharose

	Portal Vein				Intestinal Lymph			
Chemical Component	Total d 1.040–1.090 g/ml Lipoproteins	Fraction I	Fraction II	Fraction III	Total d 1.040–1.090 g/ml Lipoproteins	Fraction I	Fraction II	Fraction III
Cholesteryl ester	41.8 ± 3.6[a]	35.6 ± 2.2	37.1 ± 0.6	47.7 ± 2.4	38.4 ± 2.7	37.1 ± 1.3	37.0 ± 4.1	40.6 ± 4.3
Unesterified cholesterol	5.2 ± 0.6	5.1 ± 1.3	6.1 ± 1.2	8.0 ± 1.1	0.8 ± 0.3	0.8 ± 0.3	1.8 ± 1.3	1.7 ± 0.7
Triglyceride	0.6 ± 0.3	0.3 ± 0.2	1.1 ± 0.7	1.2 ± 1.1	4.1 ± 0.7	2.4 ± 0.7	1.8 ± 1.3	8.6 ± 3.4
Phospholipid	29.0 ± 3.5	30.0 ± 3.5	30.0 ± 2.0	22.1 ± 1.9	27.2 ± 1.0	29.0 ± 1.0	29.0 ± 0.4	18.5 ± 0.8
Protein	23.4 ± 0.5	29.0 ± 4.8	25.7 ± 1.8	21.1 ± 1.4	29.5 ± 1.9	30.7 ± 2.1	30.4 ± 1.9	30.5 ± 3.1
Esterified cholesterol / Total cholesterol	0.83 ± 0.03	0.81 ± 0.04	0.79 ± 0.03	0.78 ± 0.03	0.97 ± 0.01	0.98 ± 0.02	0.93 ± 0.06	0.93 ± 0.02

[a]Values are expressed as % of total lipoprotein mass and represent means ± SD (n = 3).

Electrophoretic studies of lipoprotein subfractions. Native lipoproteins of d 1.040–1.090 g/ml and their chromatographic subfractions were electrophoresed in two different systems. First, their specific mobility on polyacrylamide gel slabs was determined and compared to those of lipoproteins present in a normolipidemic human serum (**Fig. 3**). Under these conditions, fractions I and II of plasma or lymph origin each exhibited mobility comparable to that of human HDL, although fraction I migrated slightly further in the gel than fraction II. In samples from plasma, fraction III presented as a wide band with mobility slightly higher than that of human LDL. In contrast, this same fraction was clearly heterogeneous in intestinal lymph, showing a supplementary narrower band which migrated ahead of that shared with plasma.

The respective Stokes diameters of lipoproteins contained in chromatographic subfractions were determined by electrophoresis on 2–16% continuous gradient polyacrylamide gel slabs (**Fig. 4**). Thus, most material contained in fraction I from both portal vein plasma and intestinal lymph consistently exhibited diameters in the range of 130–160 Å (upper and lower limits of the stained bands). Lipoproteins contained in fraction II were constituted of particles with slightly greater diameters (ca. 155–165 Å). In addition, and despite the small amounts of lipoproteins available from such fractions, a supplementary population of larger lipoproteins (ca. 200–210 Å) was detectable in samples originating from plasma. Indeed, it is with regard to the content of fraction III that the most pronounced differences between portal vein plasma and intestinal lymph lipoproteins were observed. Thus, a doublet of bands with respective Stokes diameters of 250 and 260 Å (plasma), or 245 and 250 Å (lymph) was consistently observed in the two series of samples. By contrast, the presence of a supplementary population of lipoproteins corresponding to a clearly stained band of particles with diameters in the range of 190–200 Å was detected only in fraction III from intestinal lymph.

DISCUSSION

In the present studies, we have partially resolved the particle heterogeneity present within the central region of the density spectrum of plasma and lymph lipoproteins in the calf (1, 2).

In an earlier report, Cordle, Clegg, and Yeaman (21) used affinity chromatography to subfractionate a lipoprotein material obtained by preliminary gel filtration of the d < 1.21 g/ml fraction from bovine plasma, and described two populations of particles exhibiting α and β electrophoretic mobility, respectively. Use of heparin-Sepharose has allowed us to obtain an effective separation of HDL- and LDL-like particles in the d 1.040–1.090 g/ml interval in both intestinal lymph and portal vein plasma. Among

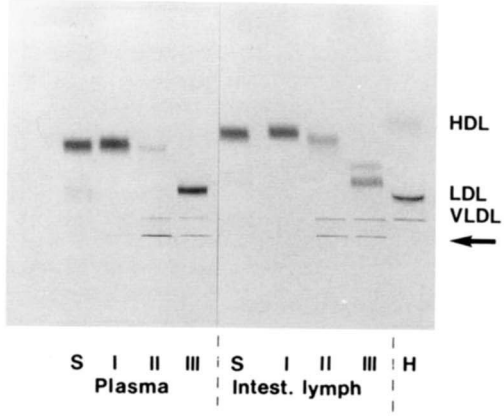

Fig. 3. A representative pattern obtained upon electrophoresis on discontinuous polyacrylamide slab gels of native d 1.040–1.090 g/ml lipoproteins from calf plasma and intestinal lymph and of their respective affinity chromatography subfractions. Arrow indicates the position of layers. Prestaining was performed using Sudan black. From left to right: total plasma lipoproteins of d 1.040–1.090 g/ml (S) and the corresponding affinity chromatography subfractions; total d 1.040–1.090 g/ml lipoproteins from intestinal lymph, and the corresponding affinity chromatography subfractions; normal human plasma (H) presenting VLDL, LDL, and HDL bands as indicated.

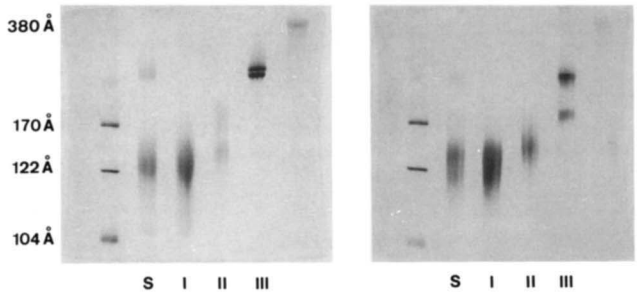

Fig. 4. Polyacrylamide gradient gel electrophoresis of native d 1.040–1.090 g/ml lipoproteins from calf plasma and intestinal lymph and of the corresponding affinity chromatography subfractions. The gel slabs contained a 2–16% monomer gradient (from top to bottom). Calibration was achieved by use of (a) a set of standard marker proteins, loaded in the first lane on the left of each slab; the standards and their Stokes diameters were respectively (from top to bottom): thyroglobulin, 170 Å; ferritin, 122 Å; and catalase, 104 Å, and (b) latex particles, (diameter 380 Å) loaded in the first lane on the right of each slab. Left part of the figure: typical results obtained in samples from portal vein plasma; right part: typical results from postprandial intestinal lymph. S: total lipoproteins contained in the d 1.040–1.090 g/ml interval; I, II, and III: corresponding affinity chromatography subfractions. Aliquots of lipoprotein protein (10 μg) were layered in each lane, except for fraction II from plasma lipoproteins, in which only 5 μg was available due to the extremely small amounts of this fraction. Gels were stained with Coomassie Brilliant blue.

the former lipoproteins, our experiments have disclosed the presence of two populations that differ in their respective concentrations, apolipoprotein contents, electrophoretic mobility, and Stokes diameters. The predominant fraction I was constituted of typical HDL. ApoA-I was the major component of its protein moiety together with small amounts of C-like peptides, while the principal lipids were cholesteryl ester and phospholipid.

The distinct elution characteristics of fraction II are most probably related to its apolipoprotein content, i.e., the presence in these lipoproteins of small amounts of a supplementary protein component with apparent $M_r \approx$ 51,000 in our gels. It is relevant here that Tall et al. (22) have presented SDS-polyacrylamide gels of proteins contained in HDL subfractions obtained from the plasma of lactating cows. These gels showed the presence of small amounts of a protein with migration characteristics resembling those of the protein with $M_r \approx 51{,}000$ noted in the present study. The precise identity of this protein remains undetermined at present.

The presence of apoE, an apolipoprotein with heparin-binding properties, has been demonstrated in a subfraction of human HDL (10). However, the M_r of apoE is consistently found within the range of 35,000–39,000 upon SDS-polyacrylamide electrophoresis (for review see ref. 23). Such values are approximately 12,000–16,000 daltons lower than the M_r of the protein component present in addition to apoA-I and C-like peptides in fraction II. Furthermore, no definitive evidence is, to date, available as to the existence of apoE in bovine species, although its presence has been suggested on the basis of electrophoretic criteria (24, 25). It is thus reasonable to assume that the protein specifically present in trace amounts in fraction II and exhibiting heparin-binding properties is distinct from apoE. It is relevant here that Borenzstajn, Kotlar, and Meredith (26), when studying rat lymph chylomicrons, demonstrated the occurrence of a quantitatively minor population of particles that bound to heparin-Sepharose. In addition to other apolipoproteins shared in common with non-heparin-binding chylomicrons, this fraction specifically contained a protein whose M_r, amino acid composition, amphiphilicity, and immunological reactivity resembled those of β_2-glycoprotein I.

β_2-Glycoprotein I has been shown by Polz and Kostner (27) to be present in all major lipoprotein classes, including postprandial chylomicrons and is a polymorphic protein with three isoforms sharing a common M_r of 54,000 (28). In our previous study of calf plasma lipoproteins (1), we reported the presence of trace amounts of a protein with $M_r \approx 54{,}000$ in lipoproteins with $d < 1.060$ g/ml. Subsequently, in our recent report on postprandial intestinal lymph lipoproteins in the calf (2), we observed the presence of a minor component with M_r 51,000–52,000 in the protein moiety of both chylomicrons and VLDL. Taking into account the different SDS-gel systems used in the two successive series of experiments, these proteins could be identical and might correspond to calf β_2-glycoprotein I. We therefore suggest that fraction II in the present study could consist of β_2-glycoprotein I-containing lipoproteins. This fraction could then represent a separate population of native, intestinally secreted HDL in the case of lymph samples. In material originating from portal vein plasma, fraction II could represent the by-product of the catabolism of a β_2-glycoprotein I-containing subpopulation of chylomicrons and, possibly, intestinal VLDL. The question then arises as to the metabolic significance of fraction II. It is conceivable that the presence of β_2-glycoprotein I, even at low concentration, entails a particular behavior of the corresponding lipoproteins, for example with regard to their ability to bind to membrane receptors and thus to compete with apoB-containing particles. Also, β_2-glycoprotein I has been demonstrated to be a potent activator of lipoprotein lipase (29). Therefore, dietary and/or metabolic factors involved in β_2-glycoprotein I synthesis and thus in the modulation of the ratio of the concentrations of fraction I/fraction II lipoproteins, both in intestinal lymph and in plasma, should be considered in future experiments.

In both physiological fluids that we have investigated, fraction III was characterized by the presence of two high M_r, apoB-like proteins. This finding is consistent with results that we reported earlier in studies on plasma and lymph lipoproteins in the calf (1, 2). Taken together, our data suggest that these two proteins would be calf counterparts to human apoB-100 and apoB-48 (20), respectively.

However, the lipoprotein material constituting fraction III in both portal vein plasma and in intestinal lymph was heterogeneous upon electrophoresis. In plasma, this phenomenon was limited to the detection of a doublet of bands with Stokes diameters in the region of 250 Å, a value typical of low-density lipoprotein particles (30). At the same time, examination of our 3% SDS gels showed that the apoB-100-like protein predominated while small amounts of the apoB-48 counterpart were also present. Thus, the distribution of the two apoB-like proteins between the two types of particles constituting the electrophoretic doublet will be the subject of future investigation.

The presence of lipoprotein [a] (Lp[a]), a lipoprotein that most probably plays a major role both in atherogenesis and in thrombosis, has been thought for many years to be restricted to humans and certain primates (for review see ref. 31). However, we have recently demonstrated the occurrence of this lipoprotein in the plasma of the hedgehog, a hibernator that appeared much earlier in the evolutionary process (11). In view of the consistent particle heterogeneity in affinity chromatography fraction III in the preruminant calf, we attempted to disclose the possible presence of Lp[a] in the plasma of this animal. However, from our experiments, three sets of arguments can be presented to discount this possibility. *1*) The respective Stokes diameters of the different lipoprotein particles present in fraction III from either plasma or lymph origin did not exceed ≈250 Å. Using the same experimental conditions, the particle diameter of authentic Lp[a] was consistently in the range of 275–285 Å, both in the hedgehog (11) and in humans (Laplaud, P. M., unpublished results). *2*) The protein moiety of Lp[a] is characterized by the presence of an apoB:apo[a] complex, with M_r variable according to the apo[a] isomorph present, but greater than ≈800,000. Dissociation of this complex is readily obtained upon reduction using agents such as β-mercaptoethanol or dithiothreitol (DTT). In the case of fraction III from either plasma or lymph in our animals, no protein component with $M_r > 800{,}000$ could be observed under nonreducing conditions, and incubation with 10 mM DTT induced no change in the apparent size of either of the two bands representative of the high M_r proteins. Finally *3*), no immunological reactivity could be observed between calf plasma, or purified fraction III of either plasma or lymph origin, and a polyclonal antiserum to human Lp[a]; this antiserum was shown earlier to give a precipitation line with Lp[a] from the hedgehog (11). Thus, for all these reasons, we suggest that Lp[a] is not a constituent of d 1.040–1.090 g/ml lipoproteins in our experimental animals.

In intestinal lymph samples, we observed a supplementary population of smaller particles, with diameters in the 190–200 Å range, and with electrophoretic mobility intermediary between those of human (or bovine) LDL and HDL particles on polyacrylamide gel slabs. As compared to our results for fraction III from plasma, appearance of this additional lipoprotein material was accompanied by *1*) a distinct increase in the intensity of the band representative of the lower M_r form of apoB in SDS gels; *2*) a sevenfold increase in the proportion of glycerides, mainly to the detriment of cholesteryl esters; and *3*) a 1.5-fold increase in the proportion of protein. However, it is not possible from our present data to exclude the possibility that some of these changes, and especially that regarding alteration in the ratio of apoB-100/apoB-48-like proteins, could be related to modifications in the composition of the two LDL-sized populations of particles constituting the slower-migrating doublet. It is of note here that the respective Stokes diameters of the lipoproteins constituting this doublet were not identical in plasma (≈250 and 260 Å) and in intestinal lymph (≈245 and 250 Å).

The question as to the protein composition of the distinct populations of lipoprotein particles eluting as fraction III from either plasma or lymph was further complicated by the observation that small amounts of a protein with size similar to apoA-I were consistently present. It is relevant in this context that subfractionation of d 1.006–1.063 g/ml plasma lipoproteins from the European badger by the same affinity chromatography methodology allowed isolation of material, denoted as fraction III, that exhibited LDL-like electrophoretic characteristics and whose protein moiety simultaneously contained apoB-100 and small amounts of apoA-I (3). It is equally relevant that, upon analysis of the protein moiety of bovine β-migrating lipoproteins purified by affinity chromatography, Cordle et al. (21) observed that "apoA-I was a minor component of this lipoprotein." In experiments conducted in the badger as well as in the calf, we cannot exclude the possibility that apoA-I in fraction III could be a contaminant resulting from incomplete or delayed elution of lipoproteins constituting the earlier part of the chromatographic profile. However, in both series of studies, the distinct electrophoretic characteristics of the first two fractions on the one hand, and of the third fraction on the other, argue against this possibility.

The electrophoretic characteristics of the supplementary lipoprotein population appearing in fraction III from intestinal lymph were intermediary between those of typical LDL and HDL. These particles most probably represent secretion products from the intestine. Furthermore, their presence may account for the increase in protein content, and possibly the simultaneous increase in that of glycerides in lymph fraction III as compared to the corresponding material originating from portal vein plasma. However, the very low concentration of fraction III (2.7 ± 0.9 mg/100 ml in our lymph samples) prevented further subfractionation and characterization of its different constituents.

The present studies have demonstrated the complexity of the lipoprotein spectrum present in the d 1.040–1.090

g/ml interval in postprandial portal vein plasma and in intestinal lymph in the preruminant calf. On the one hand, our affinity chromatography experiments permitted isolation of two populations of particles sharing the typical properties of HDL, but differing in that only one population incorporated a heparin-binding apolipoprotein. Each of these populations appeared to be homogeneous, at least on the basis of the analytical techniques that we applied. Therefore, experiments aimed at the study of the metabolism of each particle population under different dietary conditions or under the influence of drugs affecting intestinal lipoprotein metabolism are conceivable in the near future. On the other hand, fraction III from heparin-Sepharose chromatography was heterogeneous. The plurality of the lipoprotein populations contained in this latter fraction, combined with their low concentrations, represents an analytical challenge which must be overcome before further metabolic studies on each lipoprotein particle subpopulation may be contemplated.

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