papers and notes on methodology

Subfractionation of human very low density lipoproteins by heparin-Sepharose affinity chromatography

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Abstract Very low density lipoproteins obtained from normolipidemic subjects were fractionated into subclasses by means of affinity chromatography on a heparin-Sepharose column in the presence of $MnCl_2$. The four subfractions eluted at 0.05, 0.12, 0.20, and 0.38 M NaCl and they differed in chemical composition and apoprotein pattern. The relative amounts of apoB and apoE in subfractions increased with increasing concentration of the NaCl eluant. Modification of the arginyl residues with 1-2 cyclohexadione demonstrated that arginine plays an important role in determining the elution pattern of VLDL. In vitro studies indicated that only fractions eluted at 0.2 and 0.5 M NaCl compete with LDL for cellular receptors. These data suggest that the various subfractions may represent VLDL at different stages of catabolism.—**Trezzi, E., C. Calvi, P. Roma, and A. L. Catapano.** Subfractionation of human very low density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* 1983. **24:** 790–795.

Supplementary key words heparin • apoprotein B • apoprotein E

Plasma very low density lipoproteins (VLDL) are heterogeneous in size, chemical composition, and origin. The separation methods applied to isolated VLDL subfractions rely upon differences in size and hydrated density (1, 2). Shelbourne and Quarfordt introduced the separation of VLDL into two fractions by means of affinity chromatography on a heparin-Sepharose column (3). The first fraction contained very little apoE, while the second was enriched with this apoprotein. Recently Weisgraber and Mahley (4) isolated, by heparin-Sepharose chromatography, four subfractions of HDL. Two subfractions contained only apoB, one contained apoE plus other HDL apoproteins (HDL with E), and one was HDL without E. The fractions also differed in their ability to interact with cellular receptors.

Gianturco et al. (5) suggested that VLDL obtained from normolipidemic subjects is heterogenous with respect to in vitro catabolism. Only $VLDL_3$ is, in fact, able to suppress the HMG-CoA reductase activity in cultured fibroblasts, thus stressing the concept that VLDL subfractions may also differ in their metabolic fate.

In this study we report on: *1*) a method for the fractionation of VLDL by heparin-Sepharose affinity chromatography in presence of Mn^{2+}, and *2*) the partial characterization of the subfractions and their ability to compete in vitro with LDL for cellular receptors.

MATERIALS

Sepharose 4B activated with CNBr was obtained from Pharmacia (Uppsala, Sweden), and heparin (from pig intestinal mucosa) was kindly supplied by Dr. G. Prino (Crinos S.p.A., Villaguardia, Italy). Electrophoresis reagents were from BioRad (Richmond, CA). All other chemicals were analytical grade.

Abbreviations: LDL, low density lipoproteins; VLDL, very low density lipoproteins; LPDS, lipoprotein-deficient serum; CHD, cyclohexadione.

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METHODS

Plasma was obtained from fasting (12 hr) normolipidemic individuals; EDTA was used as an anticoagulant. VLDL was separated at d 1.006 g/ml by a single spin at 40,000 rpm at 12°C in a 60 Ti Rotor for 18 hr (6). It was collected and dialyzed against buffer A (0.05 M NaCl–5 mM Tris, pH 7.4).

The heparin-Sepharose column was prepared as previously described (7); free groups were blocked by addition of 1 M ethanolamine. The slurry was packed on a 2.6 × 8 cm column and equilibrated with buffer A containing 25 mM $MnCl_2$ at 4°C. Before the application to the column, $MnCl_2$ was added to the sample to a final concentration of 25 mM. VLDL was applied in a volume of 5–10 ml, allowed to equilibrate overnight at 4°C, and then eluted with three column volumes of buffer A containing 25 mM $MnCl_2$ followed by four column volumes each of buffer B (0.12 M NaCl–5 mM Tris, pH 7.4), buffer C (0.2 M NaCl–5 mM Tris, pH 7.4), and buffer D (0.5 M NaCl–5 mM Tris, pH 7.4). The choice of the stepwise elution was based on preliminary experiments using a continuous gradient of sodium chloride (from 0.05 to 0.5 M) for elution. Four peaks were eluted, at 0.05, 0.11, 0.2, and 0.38 M NaCl (**Fig. 1**). In all experiments, fractions of approximately 10 ml were collected at a flow rate of 40 ml per hr, and absorbance was monitored at 280 nm. Appropriate fractions were pooled and dialyzed for 24 hr against 0.15 M NaCl–0.01% EDTA, pH 7.4.

The reproducibility of the method was determined by elution of the same sample of VLDL in three consecutive runs; the areas under each peak were within ±6%. Furthermore, the isolated fractions were re-eluted at the same ionic strength with a greater than 90% recovery. Total recovery of protein from the column was 91 ± 3% (x ± SD, n = 6). Lipoprotein size was determined by electron microscopy of negatively stained lipoproteins, using 2% phosphotungstic acid, pH 6.8. Lipoprotein diameter was also calculated from the chemical composition of VLDL and VLDL subfractions as described by Redgrave and Carlson (8).

The protein content of lipoproteins was determined as described by Lowry et al. (9) in the presence of 0.1% sodium dodecyl sulfate to avoid turbidity. Phospholipids were determined by the method of Rouser, Siakotos, and Fleischer (10), free and esterified cholesterol by an enzymatic procedure (11), and triglycerides by a chemical method (12). The apoprotein pattern of the subfractions was estimated by SDS gel electrophoresis (13) on apoproteins delipidated by the procedure of Scanu and Edelstein (14) and solubilized in electrophoresis buffer containing 2% mercaptoethanol.

Gels were fixed with 10% TCA and stained with Coomassie blue R 250. Destaining was carried out in 2% acetic acid–20% methanol. The apoprotein distribution was estimated by scanning of the gels at 600 nm. The apoproteins were identified on the basis of their molecular weights using appropriate standards. No correction factors for the differential binding of dye to apoproteins was introduced. The area under each peak was proportional to the total amount of protein applied up to 80 μg of protein. ApoB was determined as described by Kane et al. (15) as tetramethylurea-insoluble protein. C-apoprotein distribution was determined by isoelectric focusing as previously described (16). The presence of apoB heterogeneity was assessed by SDS gel electrophoresis in a 3.8% acrylamide gel. Low molecular weight apoB was identified using chylomicrons obtained from plasma of a subject with apoC-II deficiency (17).

To study the role of arginine residues in the interaction between VLDL and heparin, arginine residues were modified with cyclohexadione (18). Control VLDL was treated in the same way with the exception that 1,2 cyclohexadione was omitted. Lipoproteins were then

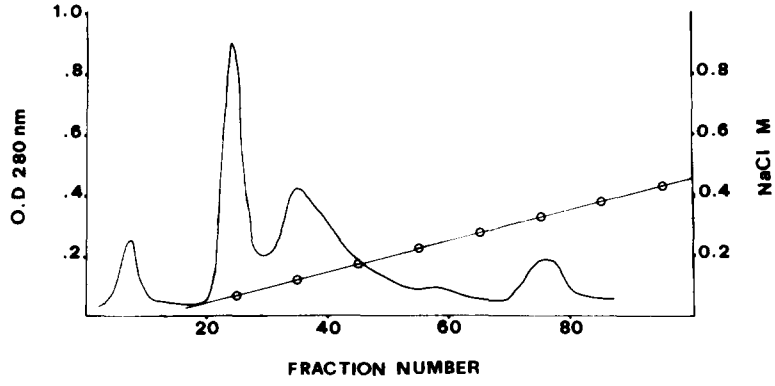

Fig. 1. Elution profile of VLDL from the heparin-Sepharose column. The elution profile was obtained using a continuous gradient of NaCl. Seven mg of lipoprotein-protein was applied in a volume of 9 ml.

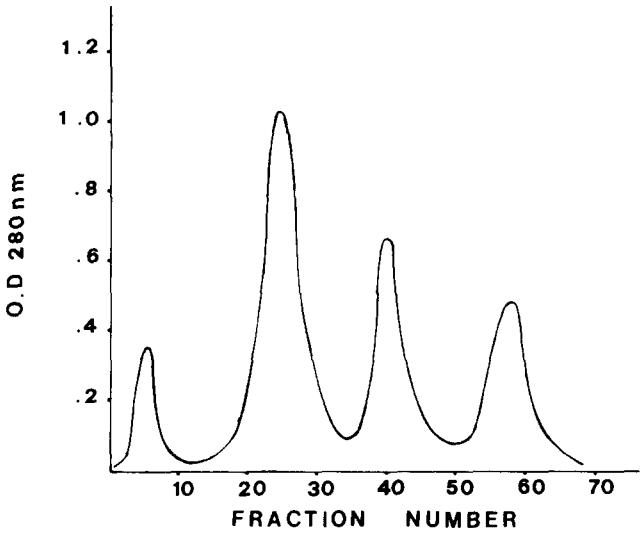

Fig. 2. Elution profile of VLDL from the heparin-Sepharose column. Seven mg of lipoprotein-protein was applied in a volume of 8.2 ml. Elution of the column was performed as described under Materials and Methods.

applied to the column and eluted as described. Quantitation of the arginyl residues modified by cyclohexadione was carried out by amino acid analysis.

For the tissue culture experiments, normal human skin fibroblasts were obtained and cultured as described (19). At the time of the experiment, fibroblasts were between the 12th and 15th passage. LDL (d 1.019–1.063 g/ml) was isolated by ultracentrifugation at 12°C for 24 hr at 40,000 rpm in a 60 Ti Rotor. The LDL was labeled with ^{125}I (20); more than 95% of the protein of LDL was apoB. Specific activity ranged from 150 to 185 cpm/ng. Cells were plated in petri dishes (3 cm diameter) and grown to about 70% confluency. The medium was changed with medium containing 5% lipoprotein-deficient serum (LPDS, d 1.250 g/ml) for 24 hr. The medium was then removed and the cell monolayer was washed with phosphate-buffered saline. Two ml of fresh medium (containing LPDS), previously chilled at 4°C, was added and the dishes were transferred to a cold room (+4°C). All subsequent steps were carried out at 4°C.

The binding assay was carried out exactly as described (19) on triplicate dishes. For the tissue culture studies, VLDL and LDL subfractions were concentrated on an Amicon cell fitted with an AMX-100 membrane at 4°C under nitrogen.

RESULTS

VLDL elution by a continuous gradient results, under our experimental conditions, in the separation of four major fractions (Fig. 1) that appeared to be fully eluted at 0.05, 0.12, 0.20, and 0.38 M NaCl. Based upon this observation, we decided to use a stepwise mode of elution using the above-mentioned concentrations of NaCl.

A representative elution pattern of VLDL from the heparin-Sepharose column using this approach is shown in **Fig. 2.** The pattern shows absorbance at 280 mm. Absorbance at 280 is not representative of mass for lipoproteins, because of light scattering. Protein measurements indicated, in fact, that only 2–8% of the total protein mass was present in the first fraction (fraction A). The size of total VLDL and VLDL subfractions was calculated from the chemical composition data (8) and determined by electron microscopy. Results (**Table 1**) showed a decreasing size of the particles from fraction A to D.

The chemical composition of the VLDL fractions is presented in **Table 2.** Fraction A contained a relatively large amount of triglyceride (more than 70% of the lipoprotein mass) and relatively low cholesterol and protein. Fraction B contained more phospholipids and protein, and slightly more free cholesterol with respect to fraction A. The same trend was observed for fraction C. A relatively high percent of the mass of fraction D was represented by esterified cholesterol plus protein. Fractions C and D had the least relative amount of triglyceride.

The analysis of the apoprotein pattern by SDS gel electrophoresis (**Fig. 3**) indicated that fraction A did not contain detectable apoE; other proteins of higher molecular weight were present as well as C peptides. In some, but not all, preparations, fraction B and C, which always contained apoE, were also enriched with a component of 64,000 molecular weight that we tentatively identified as albumin. C-apoproteins were present in all fractions; however, their relative content was low in fraction A (7.2%) and decreased from fraction B (49%) to D (28%). ApoE represented 12.8% of the total VLDL protein as determined by scanning of the gels. It was not detectable in fraction A, and was 9.7, 15, and 24% in fractions B, C, and D, respectively. It should be stressed that these data, although semi-quantitative, in-

TABLE 1. Diameter of VLDL and VLDL subfractions

	NaCl	Electron Microscopy[a]	Calculated[b]
	M	*Å*	
Total		410 (1150–230)	416 ± 82
Fraction A	0.05	701 (1362–500)	654 ± 190
Fraction B	0.12	596 (812–437)	456 ± 110
Fraction C	0.20	330 (625–280)	379 ± 98
Fraction D	0.38	290 (437–230)	300 ± 49

[a] The average diameter and size range of 100 free-standing particles is shown.

[b] Mean ± SD, n = 5.

TABLE 2. Chemical composition of VLDL subfractions[a]

	A	B	C	D	Total
Protein	4.5 ± 2.56	6.5 ± 1.16*	10.5 ± 1.93**,†	13.2 ± 2.35**,††	8.5 ± 2.6
Phospholipids	11.6 ± 2.62	15.2 ± 0.96**	17.0 ± 3.98*	21.1 ± 4.98**,†	16.5 ± 2.9
Triglycerides	75.7 ± 8.65	66.2 ± 8.82*	52.7 ± 7.80**,†	48.8 ± 7.24**,††	58.2 ± 9.7
Free cholesterol	5.1 ± 1.64	7.8 ± 3.50	6.4 ± 2.68	6.6 ± 2.54	6.4 ± 1.6
Cholesteryl esters	4.2 ± 1.80	6.4 ± 2.28	12.9 ± 3.36**,††	9.2 ± 3.10*,†	10.4 ± 2.2

[a] Values expressed as means ± SD, n = 5.
*, $P < 0.05$ vs. A; **, $P < 0.01$ vs. A; †, $P < 0.05$ vs. B; ††, $P < 0.01$ vs. B.

dicate a trend towards a relative increase of apoE from fraction B to D. ApoB content was 29% of protein in VLDL and 23, 26, 31, and 41% in fractions A, B, C, and D, respectively, as determined by the tetramethylurea assay (Fig. 3, **Table 3**). Further analysis of the apoC distribution among different subfractions by isoelectric focusing revealed no changes in the relative content of apoC-II and C-III subspecies (three experiments, data not shown). ApoB-48 was not detectable in VLDL or in VLDL subfractions by SDS gel electrophoresis (not shown).

Modification of arginyl residues affected the elution pattern of VLDL. After treatment with 1,2-cyclohexadione, two main fractions were recovered (**Fig. 4b**). The same lipoprotein treated as control showed four peaks (Fig. 4a). Quantitation of the arginyl residues modified indicated that about 50% of the arginyl residues was modified in total VLDL as well as in fraction A and B. Moreover, SDS gel electrophoresis of fraction A and B obtained after CHD modification of VLDL indicated that both fractions contained apoE (not shown).

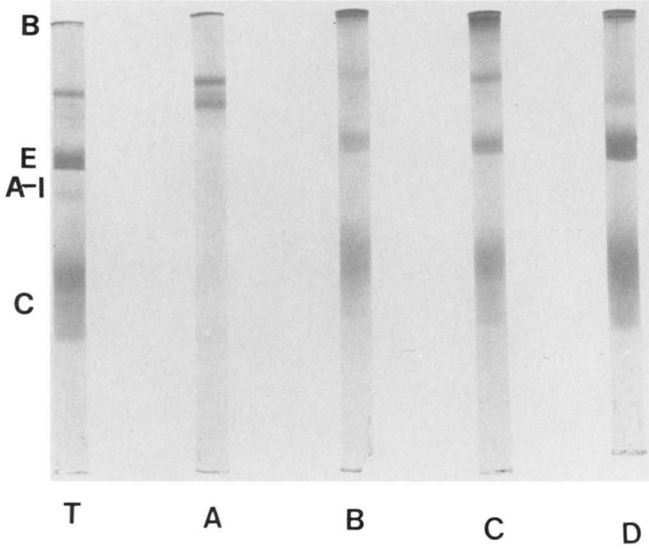

Fig. 3. SDS gel electrophoresis of VLDL (T) and VLDL subfractions; 80, 45, 60, 69, and 89 μg of protein were applied to the gels. Apolipoproteins B, E, A-I, and C were identified using purified proteins as standards.

In vitro total VLDL was only slightly effective in competing with LDL for binding to cellular receptors (15% of the LDL activity). Fractions A and B were ineffective. Fraction C, at 120 μg of protein/ml, reduced binding by 30%, while fraction D was the most effective (**Fig. 5**).

DISCUSSION

The interaction between heparin and apoE is well known and offers a tool for separating subfractions of HDL_2 and VLDL that differ in apoprotein pattern and, possibly, metabolic properties (3, 4). Shelbourne and Quarfordt reported the separation of two VLDL subfractions by heparin-Sepharose affinity chromatography in the absence of divalent ions (3). In this report we describe a method of separation of VLDL using a heparin-Sepharose affinity column in the presence of Mn^{2+}. This method is reproducible and allows a protein recovery of 91%. Fractions were stable upon rechromatography and were re-eluted at the same molarity of NaCl (more than 90%, data not shown).

The chemical composition of the fractions (Table 2) suggests that fractions A and B should contain relatively large VLDL while C and D should be of smaller size (14). The calculated diameter of the subfractions and of total VLDL was in good agreement with the data obtained by electron microscopy (Table 1), demonstrating that progressively smaller fractions are eluted from the column. This finding is also in agreement with the data on the apoprotein pattern of fractions B, C, and D, i.e., an increased percent content of apoB and E and

TABLE 3. Distribution of apoproteins B, C, and E among VLDL subfractions

	A	B	C	D	Total
			% of total protein		
ApoB	23.7	26.4	31.2	45.0	29.1
ApoE	ND	9.7	15.4	24.1	12.8
ApoC	7.2	49.2	39.3	27.6	44.1

ND, not detectable; x = 3.

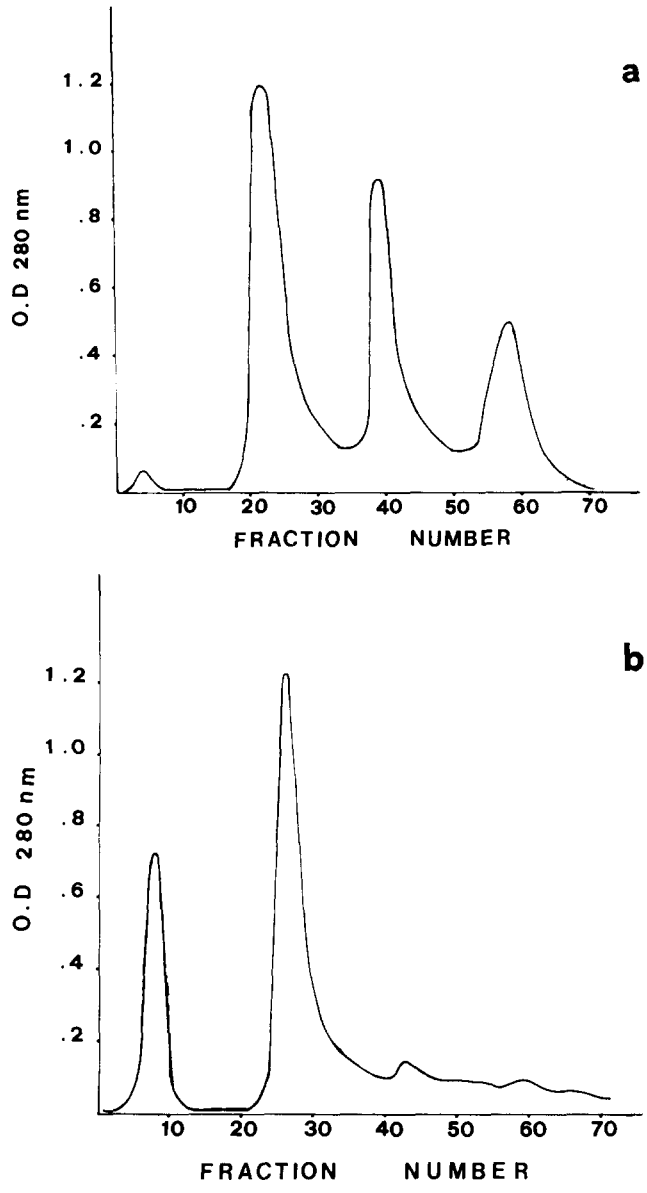

Fig. 4. a) Elution profile of control VLDL (see Materials and Methods). Nine mg of protein was applied in a volume of 10 ml. b) Elution profile of VLDL modified with 1,2-cyclohexadione. Six mg of protein was applied to the column in a volume of 10 ml.

a decreasing amount of C-apoproteins (15). Fraction A, however, contained a small amount of C-apoproteins and no apoE. Our results are at variance with those of Shelbourne and Quarfordt who reported that the unbound fraction accounts, at least by absorbance, for a large proportion of VLDL. The protein content as % of the mass was not reported; however, high molecular weight proteins were present in the unbound fraction as determined by SDS gel electrophoresis (3).

The content of apoE and apoB in the different subfractions appears to depend upon the ionic strength required to elute the fractions (the higher the ionic strength the higher the apoE and B content) and is consistent with the concept that both apoB and apoE can interact with heparin. Fraction A contains no detectable apoE. The possibility that this fraction preferentially dissociates apoE upon chromatography under the conditions described must be considered. This, however, is unlikely since we eluted the column, after several runs of VLDL, with 2 M NaCl in presence of 8 M urea and were unable to find any apoE eluted from the column.

Modification of arginyl residues reduced the interaction between VLDL and heparin resulting in an increase of fractions A and B and a great reduction of fractions C and D. This is in agreement with the reduction of the interaction between low density lipoproteins and heparin reported after cyclohexadione modification (21) and suggests that arginine residues may play a role in the fractionation of C and D, while some other factors (phospholipids) may influence the separation of fractions A and B. It should also be stressed that our data do not allow an evaluation of the roles of apoB and apoE in determining these subfractions. The observation that both apoB and apoE increase from fraction B to D suggests that both apoproteins are involved, although preliminary data using LDL indicate that, under our experimental conditions, LDL (apoB) is eluted at high ionic strength. Further experiments are required to address this question.

The data on the competition of VLDL and VLDL fractions for the binding of LDL to cellular receptors on human skin fibroblasts cultivated in vitro demon-

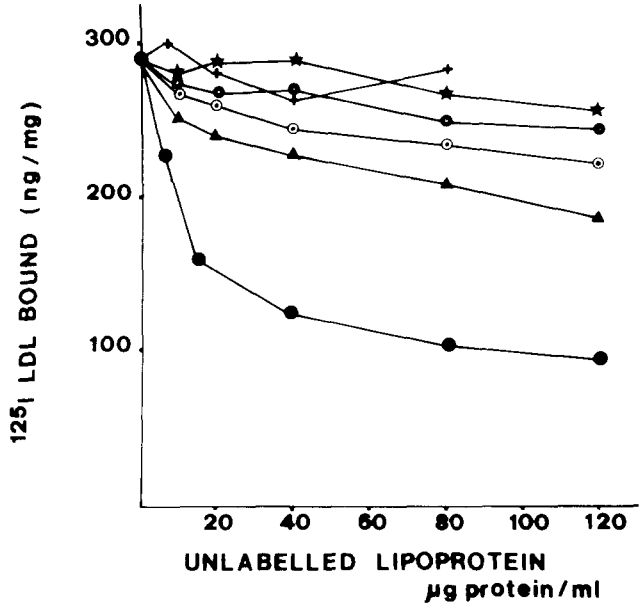

Fig. 5. Competition of unlabeled lipoproteins with ^{125}I-LDL (185 cpm/μg, 10 μg/ml) for binding to cellular receptors. ● —— ●, LDL; ❶ —— ❶, total VLDL; + —— +, VLDL, fraction A; ★ —— ★, VLDL, fraction B; ⊙ —— ⊙, VLDL, fraction C; ▲ —— ▲, VLDL, fraction D. Data presented are the means of triplicate determinations that did not differ more than 10%.

strate that total VLDL shows a modest effect while fraction C and fraction D compete with LDL. Gianturco et al. (5) showed that total VLDL from normolipidemic subjects is relatively ineffective in suppressing the HMG-CoA reductase activity in cultured fibroblasts, while $VLDL_3$ isolated by ultracentrifugation is much more effective.

The data on chemical composition, apoprotein pattern, and size of subfractions indicate that a similarity exists between fractions C and D and $VLDL_3$ and are consistent with the data obtained in tissue culture experiments. If the in vitro data also hold true in vivo, these VLDL fractions may be catabolized also by receptor-mediated pathways in vivo, and could represent VLDL that has been partly catabolized.

The possibility that fraction D represents IDL must also be considered. Incomplete separation of IDL from VLDL by ultracentrifugation has been reported (5); the chemical composition data, diameter, and apoprotein pattern of fraction D, however, indicate that this fraction is not an IDL.

In conclusion, the method described here is reproducible and allows the separation of VLDL subfractions that differ by chemical composition and apoprotein pattern. Furthermore, sequential ultracentrifugation can be avoided. The fractions also differ in their ability to interact with cellular receptors in vitro. This method may therefore be applied to the separation of VLDL into fractions that are metabolically distinct in vitro and, in vivo, may represent different stages of VLDL catabolism.

Further work, however, is required to clarify the extent to which apoB and apoE are responsible for the interaction of VLDL with heparin and to fully understand the physiological significance of these subfractions.

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