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Subpopulations of Human Serum Very Low Density Lipoproteins†

Edward Pearlstein‡ and Frederick Aladjem*

ABSTRACT: Very low density human serum lipoproteins (VLDL, *d* 0.98–1.006 gm/cm³) have been found to be composed of at least four distinct subpopulations of molecules, which differ with respect to protein and polypeptide composition. Differential preparative ultracentrifugation, electrophoretic, and immunological techniques were used to detect and partially characterize the subpopulations. The composition of subpopulations (LDL = low density lipoproteins, polypeptides are designated by their C-terminal amino acids except R-x which has a blocked C-terminal amino acid) is: (1) LDL, R-Ala, R-Thr, R-Gln, R-x; (2) LDL,—, R-Thr, R-Gln, R-x; (3) LDL, R-Ala, —, —, R-x; (4) LDL, R-Ala, R-Thr, —, —. In addition, evidence is presented which indicates the existence of a subpopulation which does not react with anti-LDL antiserum.

Various physical and chemical data indicate that VLDL[1] as a class is heterogeneous. The heterogeneity of VLDL is exemplified by a broad S_f range of 20–5000 (Gofman *et al.*, 1954), particle size variations from 275 to 2592 Å (Gustafson *et al.*, 1965) and widely different ratios of lipid to protein (Rodbell, 1958).

In previous studies, it was shown that HDL-2 and HDL-3 are each composed of at least two subpopulations of molecules, one, approximately 90%, which contains both R-Gln and R-Thr, and the other, approximately 10%, which does not contain R-Gln but does contain R-Thr (Borut and Aladjem, 1971; Albers and Aladjem, 1971).

The present investigation was undertaken to ascertain whether subpopulations which differ with respect to polypeptide composition also occur among VLDL.

It was found that VLDL is composed of at least four subpopulations which differ with respect to protein and polypeptide composition, and of an additional subpopulation, which does not react with anti-LDL antiserum.

Materials and Methods

Isolation and Characterization of VLDL. Fresh pooled human serum was obtained from the blood bank. Each pool consisted of the serums of several hundred donors. VLDL was isolated from a given pool as previously described (Pearlstein *et al.*, 1971) except that the final dialysis against distilled water was omitted.

Hydrated density classes of VLDL were isolated by the method of Gustafson *et al.* (1965). The hydrated density classes were characterized by their S_f ranges as follows: B (S_f 400–5000), C (S_f 100–400), D (S_f 50–100), and E (S_f 20–50).

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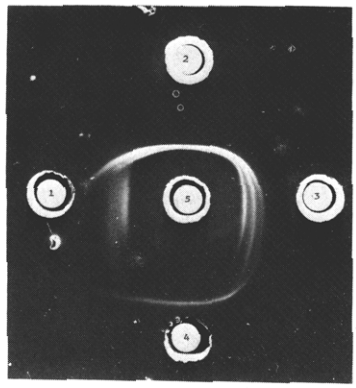
[1] Abbreviations used are: VLDL, very low density lipoproteins (*d* 0.98–1.006 g/cm³); LDL, low density lipoproteins (*d* 1.025–1.050 g/cm³); HDL, high density lipoproteins (*d* 1.075–1.195 g/cm³); polypeptides are designated by their C-terminal amino acids as R-Thr, R-Gln, R-Val, R-Glu, and R-Ala except R-x which has a blocked C-terminal amino acid.



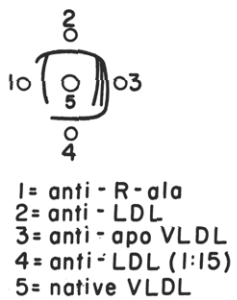
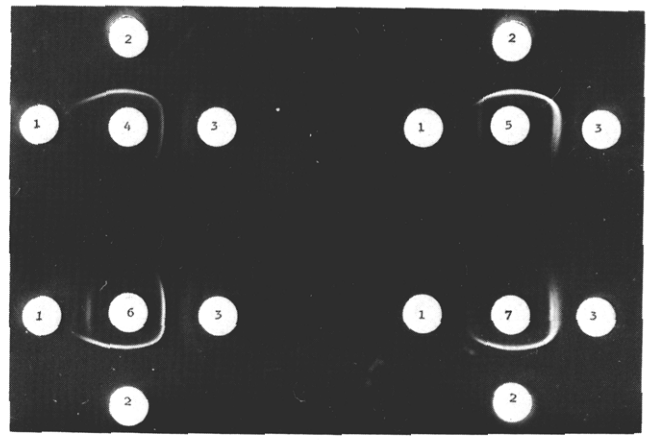


FIGURE 1: Immunodiffusion results of VLDL (center well) reacting with (1) anti-R-Ala, (2) anti-LDL, (3) anti-apoVLDL, and (4) anti-LDL (diluted 1:15) (a, left). Schematic representation of the results of part a (b, right).



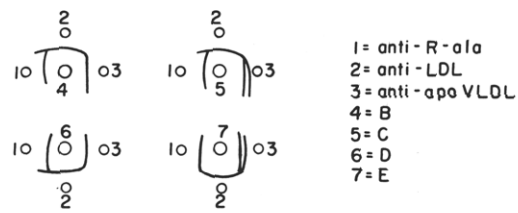


FIGURE 2: Immunodiffusion results of ultracentrifugal fractions of VLDL: (1) anti-R-Ala, (2) anti-LDL, (3) anti-apoVLDL, (4) B, (5) C, (6) D, and (7) E (a, top). Schematic representation of the results of part a (b, bottom).

The S_f ranges were confirmed in the analytical ultracentrifuge using the Schlieren optical system. For isoelectric focusing, VLDL was used without further purification.

Delipidation. VLDL was delipidated using a modification of the method of Albers and Scanu (1971) as follows. One volume of native VLDL in 0.9% saline at a concentration of about 5 mg of protein/ml was added to ten volumes of cold ethanol–ether (3:1, v/v). The mixture was continuously extracted for 5–6 hr at 7°. At the end of this time, the coarse precipitate was centrifuged, the supernatant discarded, and the precipitate resuspended in cold ethanol–ether (3:2, v/v). The extraction proceeded for an additional 16–18 hr, after which the precipitate was centrifuged, washed twice in cold ether, and dried under a stream of N_2. The solid apoprotein was redissolved in 0.01 M Tris-HCl (pH 8.2), containing 8 M urea. Only that portion of the solid apoprotein soluble in this buffer was used and will hereafter be referred to as apoVLDL. Partially delipidated VLDL was obtained by one ether extraction (v/v) of VLDL at 7° for 18 hr. This procedure removed the neutral lipids and left the LDL protein soluble in the aqueous phase (Avigan, 1957).

Preparation of Antisera. Rabbit antisera to the HDL polypeptides R-Thr, R-Gln, and R-x were those characterized and used previously (Borut and Aladjem, 1971). Anti-LDL was produced against purified S_f 4–8 lipoprotein (Aladjem *et al.*, 1957). It did not react with HDL. The anti-R-Ala was produced against VLDL R-Ala; it did not cross-react with HDL polypeptides R-Thr, R-Gln, R-x, nor with R-Glu obtained from VLDL. The anti-apoVLDL was also produced in rabbits. This antiserum did not react with LDL.

Isoelectric Focusing. Prior to use, the LKB column was coated with dimethyldichlorosilane (Bio-Rad). Native VLDL was exhaustively dialyzed against 33% ethylene glycol (Kostner *et al.*, 1969) and added to the column in a linear sucrose gradient (LKB Instruction Manual) of from 50 to 0% containing 33% ethylene glycol. Carrier ampholytes (Vesterberg and Svensson, 1966), in the pH range 4–6, were added to give a final ampholyte concentration of 1–2%. Lipoprotein (30 mg) was electrofocused at 10° for 60–72 hr. The voltage was gradually raised, over a period of 10 hr, to a final voltage of 1500 V and a current of about 5 mA.

After completion of the run, 1-ml fractions were collected and the pH and optical density (280 nm) of each were determined. The pH was read at 21° after diluting the sample 1:1 with distilled water, using a Radiometer 25 pH meter and the GK 2302C electrode.

^{125}I Labeling. The native VLDL was labeled with ^{125}I according to the method of McFarlane (1958) as described by Albers and Aladjem (1971). After exhaustive dialysis against 0.05 M Tris-HCl (pH 8.2) containing 0.02 M KI, followed by 0.05 M Tris-HCl (pH 8.2), the sample was passed through a Sephadex G-25 column (0.9 × 20 cm) prior to use. The trichloroacetic acid precipitable radioactivity was about 90%.

Immunochemical Procedures. Immunodiffusion was carried out using 1% agarose gels in 0.05 M Tris-HCl (pH 8.2). Precipitation of labeled VLDL by specific antisera was performed as previously described for the precipitation of labeled HDL (Albers and Aladjem, 1971). Tris-HCl buffer (pH 8.2) was used throughout. All precipitation reactions were carried out in antibody excess.

Polyacrylamide Gel Electrophoresis. The method was essentially that of Davies (1964). Acrylamide (Eastman) in monomer concentration of 7.5% was used; not more than 3 mA/tube (6 × 100 mm) was applied during the run.

Results

Preparative Ultracentrifugation. Different hydrated density classes of VLDL were isolated from three serum pools by preparative ultracentrifugation as described. Analytical ultracentrifugation experiments confirmed the stated S_f distribu-

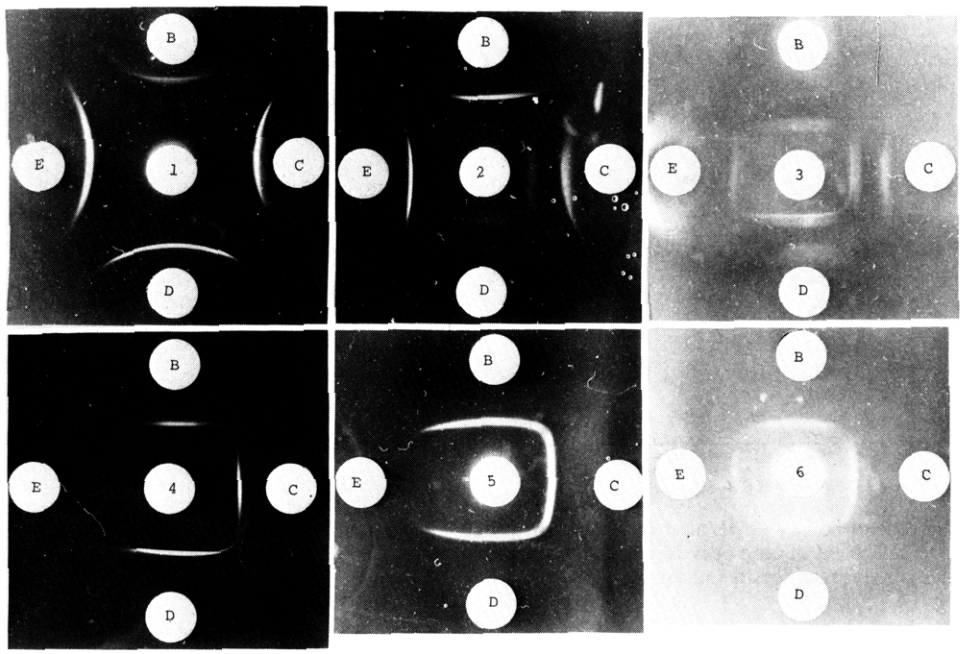


FIGURE 3: Immunodiffusion results of partially delipidated density classes B, C, D, and E reacting with (1) anti-LDL, (2) anti-apoVLDL, (3) anti-R-Ala, (4) anti-R-Thr, (5) anti-R-Gln, and (6) anti-R-x.

tions. Immunodiffusion experiments were carried out to ascertain whether immunologic differences exist between the different hydrated density classes of VLDL. The results given were consistent for all preparations employed.

Figures 1 and 2 show the results of native VLDL and of each of the four hydrated density classes reacting with the following antisera: anti-LDL, anti-R-Ala, and anti-apoVLDL. Figure 3 shows the reaction of the same four hydrated density classes of VLDL after partial (ether) delipidation, with the same antisera, as well as with anti-R-Thr, anti-R-Gln, and anti-R-x.

In Figure 1 the partial identity reaction between wells 1 and 2 is interpreted to indicate that native VLDL contains two subpopulations, one of which contains LDL antigen and R-Ala (subpopulation 1), and the other, which contains LDL antigen but not R-Ala (subpopulation 2). The fusion of lines between wells 2 and 3 and the multiple zones between the center well and well 3 are interpreted to indicate that VLDL consists of several immunochemically distinct subpopulations (multiple lines), each of which contains the LDL antigen (fusion with LDL).

Since (a) the spur mentioned above formed toward the anti-R-Ala well (well 1) and there was no spur toward the anti-apoVLDL well (well 3), and (b) the reaction of native VLDL with anti-R-Ala produces one zone of precipitation, while its reaction with anti-apoVLDL produces multiple zones of precipitation, it follows that other Tris–urea soluble polypeptides, besides R-Ala, are found on the surface of native VLDL. Since native VLDL does not precipitate with anti-R-Thr, anti-R-Gln, or anti-R-x (unpublished results), the reaction of identity between wells 2 and 3 and multiple zone formation between VLDL and anti-apoVLDL must be due to antibody against one of the other polypeptides found in the Tris–urea soluble portion of delipidated VLDL, such as R-Val or R-Glu (Brown *et al.*, 1969, 1970). The presence of these polypeptides on the surface of native VLDL is therefore postulated.

In Figure 2 are the results of each of the different hydrated density classes of VLDL reacting with the indicated antisera. Hydrated density classes B, C, and D react identically with whole VLDL. With E, however, the spur between wells 1 and 2 is missing. No spur could be detected even when E was allowed to react at high concentration. We conclude that subpopulations 1 and 2 are present in B, C, and D but subpopulation 2 is absent from E.

Unfractionated VLDL reacts with anti-apoVLDL to form three zones of precipitation (Figure 1). As seen in Figure 2, B and D give one zone with this antiserum, and C and E each form two zones.

In Figure 3 are given the reactions of B, C, D, and E after partial delipidation with ether, with each of the six antisera. Anti-LDL, anti-apoVLDL, anti-R-Ala and anti-R-x precipitate with each of the classes. Only B, C, and D precipitate with anti-R-Thr and anti-R-Gln; E does not react with either of these antisera. It is concluded that E contains a subpopulation, subpopulation 3, which contains LDL, R-Ala, and R-x but not R-Thr or R-Gln.

The zones of precipitation in Figure 3 show two types of curvature: one zone is essentially a straight line, the other zone is convex toward the antibody well. Based upon the curvatures of the zones of precipitation (Aladjem *et al.*, 1959), it is obvious that each class contains at least two species of molecules. The diffusion coefficient of one of these is approximately that of antibody; the diffusion coefficient of the other is much less than that of antibody and this material is therefore probably of much higher molecular weight. For simplicity, we will call these species L-MW (low molecular weight) and H-MW (high molecular weight) species, respectively.

It can be seen from Figure 3 that B, C, D, and E each contain both H-MW and L-MW. Only H-MW reacts with anti-LDL; L-MW does not. Both H-MW and L-MW react with anti-apoVLDL. Heterogeniety (multiple zones of precipitation) occurs in L-MW. There was only a very weak reaction between H-MW of B, C, and D and anti-R-Ala. No reaction

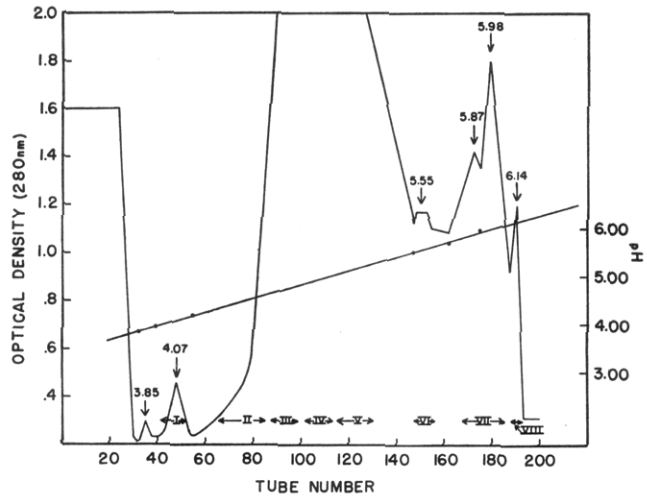


FIGURE 4: Elution profile of native VLDL after isoelectric focusing in 33% ethylene glycol. The pH of each peak is given, and the tubes which were pooled to obtain fractions are indicated.

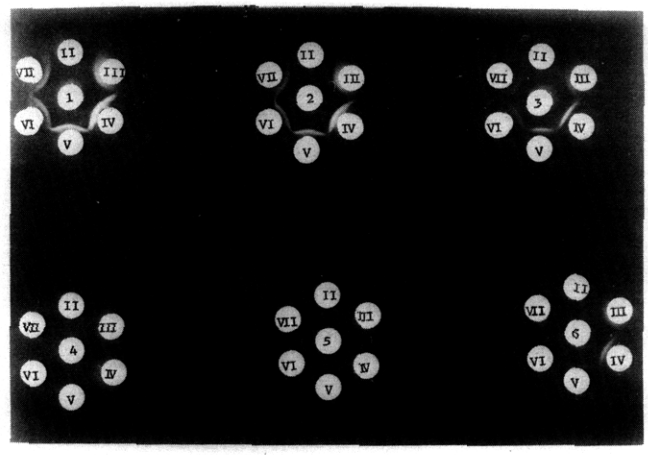


FIGURE 5: Immunodiffusion results of isoelectric focusing fractions of VLDL reacting with (1) anti-LDL, (2) anti-apoVLDL, (3) anti-R-Ala, (4) anti-R-Thr, (5) anti-R-Gln, and (6) anti-R-x.

was observed between H-MW of E and anti-R-Ala. Multiple zones were observed between L-MW of B, C, D, and E and anti-R-Ala, but the reactions were weak. Judging from the location of the zones of precipitation, however, it is apparent that the reason for the faint zones was not low antibody content, but low R-Ala content in each of the VLDL density classes.

Only the L-MW component reacts with anti-R-Thr, anti-R-Gln and anti-R-x; however, E does not react with anti-R-Thr and anti-R-Gln. The double line between C and anti-R-Thr suggests that there are at least two immunologically distinct subpopulations, both containing R-Thr.

Isoelectric Focusing. Figure 4 shows the elution profile of native VLDL. When the column was run a second time, minor variations in the p*I* for the peaks were obtained. However, the immunological characteristics and polyacrylamide gel patterns given below are consistent for both columns. The elution profile indicates that VLDL contains molecules with a broad distribution of isoelectric points. The eluted protein was arbitrarily divided into eight fractions as shown.

Sedimentation coefficients of isoelectric focusing fractions were determined in the analytical ultracentrifuge. As a control, an unfractionated VLDL preparation was dialyzed against 33% ethylene glycol and subsequently against the same buffer as the fractions. The results are given in Table I. An inverse relationship between p*I* and average S_f rate was observed for all fractions.

Native VLDL and isoelectric focusing fractions were then analyzed by immunodiffusion to ascertain whether any differences in immunological reactivity exist between VLDL and the fractions.

The reactions of native VLDL with various antisera are given in Figure 1. The reactions of isoelectric focusing fractions with the same antisera, as well as with anti-R-Thr, anti-R-Gln and anti-R-x are given in Figure 5. The following similarities were observed: the reaction of anti-LDL and anti-apoVLDL with VLDL and the fractions produces multiple zones of precipitation; anti-R-Ala reacts with these antigens to produce one zone of precipitation. Neither anti-R-Thr nor anti-R-Gln react with the fractions or VLDL. The following differences were observed: a weak line of precipitation developed between fractions III, IV, and V and anti-R-x; anti-R-x does not react with native VLDL. These reactions with anti-R-x were the only difference observed between native VLDL and isoelectric focusing fractions.

The isoelectric focusing fractions were then totally delipidated and the apoprotein of each was run on polyacrylamide gel electrophoresis. The polyacrylamide gel patterns for the slow migrating polypeptides are shown in Figure 6. Differences in polypeptide distributions, not attributable to concentration differences, can be seen. The fast migrating polypeptides were present but they were difficult to detect. A fraction of the fast polypeptides was probably lost during dialysis, and, therefore, these polypeptides were not used in the classification of subpopulations and are not indicated in Figure 6. Fractions I, II, and III contain more R-Gln than R-Thr.

TABLE I: Isoelectric Points and Sedimentation Coefficients of VLDL Fractions Obtained by Isoelectric Focusing.

VLDL	I	II	III	IV	V	VI	VII	VIII
p*I*	4.07	4.50	4.75	4.95	5.15	5.55	5.93	6.14
S_f[a] 66	Nd	98	82	66	44	Nd	38	Nd

[a] Ultracentrifuge analyses were done at *d* 1.063 g/cm³, 20°. Protein concentrations were approximately 1 OD (280 nm). S_f rates were determined according to Schachman (1957).

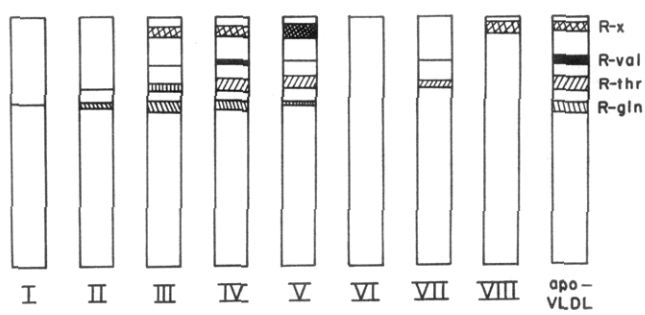


FIGURE 6: Polyacrylamide gel electrophoresis, in 8 M urea, of delipidated isoelectric focusing fractions of VLDL. The closely cross-hatched area at the top of gel V represents a high concentration of the R-x peptide. The fast-migrating polypeptides are not shown.

TABLE II: Precipitation of [^{125}I]VLDL with Antisera.

Reaction Mixture	Cpm[a,b] Total	Cpm[a,b] Ppt	% of Counts Pptd[a,b]	Cumulative Counts Pptd
(A) 20 μl of [^{125}I]VLDL and 0.3 ml of anti-apoVLDL	23,493 ± 153	16,821 ± 130	71.6 ± 2.5	
(B) 100 μl of supernatant of A and 0.3 ml of anti-LDL	2,198 ± 47	140 ± 12	6.3 ± 2.4	73.4[c]
(C) 20 μl of [^{125}I]VLDL and 0.3 ml of anti-LDL	24,270 ± 156	14,197 ± 119	58.5 ± 3.2	
(D) 100 μl of supernatant of C and 0.3 ml of anti-apoVLDL	3,331 ± 58	939 ± 31	28.2 ± 2.2	70.2
(E) 20 μl of [^{125}I]VLDL and 0.2 ml of anti-R-Ala	23,859 ± 154	9,377 ± 97	39.3 ± 1.4	
(F) 20 μl of [^{125}I]VLDL and 0.3 ml of nonimmune serum	24,332 ± 156	681 ± 26	2.8 ± 0.2	

[a] Values are an average of eight determinations on [^{125}I]VLDL isolated from two different pools. [b] Average ± std dev. [c] The value was determined as follows: 6.3(100 − 71.6) = 1.8%; 1.8% + 71.6% = 73.4%.

Fraction IV appears to contain equal amounts of R-Gln and R-Thr. Fraction V contains much less R-Gln than R-Thr. Fraction VII seems to be entirely devoid of R-Gln. Fractions V and VII contain more R-x than the other fractions.

Precipitation of [^{125}I]VLDL with Antisera. Precipitin analysis was performed using [^{125}I]VLDL and several antisera. As stated under Methods, rigorous controls were always carried out to make sure that reactions were always in antibody excess. The antisera and the amounts of radioactivity precipitated are indicated in Table II. It was found that anti-apoVLDL precipitates 71.6% of the total radioactivity, anti-LDL 58.5%, anti-R-Ala 39.3%, and non-antibody-containing control serum 2.8%.

Goat anti-rabbit γG was added to supernatants to check for the presence of soluble antigen–antibody complexes and for complexes with a hydrated density low enough to float during centrifugation. This indirect precipitation, indicative of the total amount of bound antigen in the supernatant, yielded only an additional 2–5% precipitate.

Sequential precipitation (Table II) of [^{125}I]VLDL with anti-apoVLDL followed by anti-LDL precipitated 73.4% of the radioactivity. The reverse sequence of antisera, anti-LDL followed by anti-apoVLDL, precipitates 70.2% of the radioactivity.

Discussion

VLDL encompasses a broad hydrated density range of lipoproteins. Although it is known that the variations in density are due to differences in lipid to protein ratio, it has never been shown that subpopulations with different protein moieties exist within VLDL. The results just described provide evidence for the existence of at least four distinct subpopulations, each with a different protein and/or polypeptide composition. An additional subpopulation is distinguished by its reactivity with various antisera.

Subpopulations 1 and 2. These subpopulations were identified in unfractionated VLDL (Figure 1). By immunodiffusion, only one zone of precipitation was obtained between VLDL and anti-LDL, and anti-R-Ala. At the intersection of the zones, a spur formed toward the anti-R-Ala well, indicating the presence of VLDL molecules containing LDL antigen but no R-Ala polypeptide (subpopulation 2). Since a spur did not form toward the anti-LDL well, molecules that have R-Ala polypeptide on the surface must also have LDL antigen present (subpopulation 1). After partial delipidation, these subpopulations were shown by immunodiffusion to contain R-Thr, R-Gln, and R-x. Subpopulations 1 and 2 were found in hydrated density classes B, C, and D but were not detected in hydrated density class E (Figure 2).

Subpopulation 3. In the hydrated density class E, a different subpopulation was distinguished by immunodiffusion. Figure 3 shows that the subpopulation contains LDL antigen, R-Ala, and R-x, but does not contain R-Thr or R-Gln.

Subpopulation 4. Fractions of VLDL were isolated by isoelectric focusing. One of these, fraction VII, was shown by a combination of immunodiffusion and polyacrylamide gel electrophoresis to contain a distinct protein and polypeptide distribution. Figure 5 demonstrates the presence of LDL antigen and R-Ala in this fraction. Polyacrylamide gel electrophoresis (Figure 6) shows the presence of R-Thr; R-Gln and R-x could not be detected.

Subpopulation 5. Precipitation of [^{125}I]VLDL with antisera (Table II) gave the following results: anti-apoVLDL precipitated 71.6% of the total counts while anti-LDL only precipitated 58.5%. Sequential precipitation of [^{125}I]VLDL with both antisera (Table II) yielded similar percentages of radioactivity precipitated. We conclude that there must exist molecules which contain the Tris–urea soluble polypeptides which constitute the apoVLDL antigen, but that these molecules do not have the LDL antigen accessible for antibody binding. This describes subpopulation 5.

The existence of subpopulation 5 was not expected since the results of immunodiffusion with native VLDL did not reveal any spur formation between VLDL reacting with anti-apoVLDL and anti-LDL. There are two possible explanations for this: (1) that sufficiently large amounts of antisera were used in the immunodiffusion plates to solubilize a small spur that might have formed and thus give the appearance of an "identity reaction," (2) it is conceivable that native VLDL undergoes some sort of structural change while diffusing through 1% agarose and this is unmasking LDL determinants.

TABLE III: Composition and Distribution of Subpopulations of VLDL.

Subpopulation	Composition	Hydrated Density Classes				Isoelectric Fractions					
		B	C	D	E	II	III	IV	V	VII	VIII
1	LDL, R-Ala, R-Thr, R-Gln, R-x	+[a]	+	+	−	+	+	+	+	−	−
2	LDL, —, R-Thr, R-Gln, R-x	+	+	+	−	Nd	Nd	Nd	Nd	−	−
3	LDL, R-Ala, —, —, R-x	Nd	Nd	Nd	+	−	−	−	−	−	+
4	LDL, R-Ala, R-Thr, —, —	Nd	Nd	Nd	−	−	−	−	−	+	−

[a] + = subpopulation present; − = subpopulation absent; Nd = not determined.

Table III summarizes the composition and distribution of subpopulations of VLDL. A total of four distinct subpopulations with different protein compositions have been resolved. Subpopulations were determined on the basis of the presence or absence of a particular protein or polypeptide. No differentiation was attempted on the basis of amount of protein or polypeptide present. The four subpopulations represent a minimum. Further subpopulations are likely to be discovered.

The meaning of this diversity of protein composition among native VLDL molecules remains unknown. Perhaps different subpopulations of VLDL will be found to have different functional roles, and/or perhaps the site of synthesis of the subpopulation is different. It is known that the majority of VLDL is produced in the liver (Radding and Steinberg, 1960), but the intestine is also a site of VLDL synthesis (Windmueller and Levy, 1968). A dynamic relationship between serum levels of VLDL and HDL exists (Levy *et al.*, 1966). Subpopulations of HDL recently discovered (Albers and Aladjem, 1971) may also be a contributing factor to the diversity of the protein composition of native VLDL molecules.

In vitro and *in vivo* polypeptide transfer from VLDL to HDL has recently been demonstrated (Bilheimer *et al.*, 1971). It will be interesting to see which subpopulation of VLDL is involved in this type of reaction. Since R-Ala was one of the peptides transferred, subpopulation 2 cannot be involved, unless subpopulation 2 is generated from subpopulation 1 by the loss of R-Ala. These as well as many other questions need to be investigated.

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