Precipitation of ^{125}I-Labeled Lipoproteins with Specific Polypeptide Antisera. Evidence for Two Populations with Differing Polypeptide Compositions in Human High Density Lipoproteins*

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ABSTRACT: R-Thr and R-Gln polypeptides in high density lipoproteins HDL2 and HDL3, very high density lipoproteins (VHDL), and very high density lipoproteins generated from HDL2 and HDL3 (GVHDL) were quantitatively precipitated by polypeptide specific anti-R-Thr and anti-R-Gln antisera. The results of quantitative precipitation of labeled HDL2, and HDL3, were as follows: 93–97% of the radioactivity was precipitated by (a) anti-HDL2 or anti-HDL3 followed by anti-γ-globulin, (b) anti-R-Thr followed by anti-γ-globulin, or (c) anti-R-Gln followed by anti-R-Thr followed by anti-γ-globulin; only 84–90% of the radioactivity was precipitated by anti-R-Gln followed by anti-γ-globulin. Anti-R-Thr precipitated 95–97% of the radioactivity of ^{125}I-labeled GVHDL, whereas anti-R-Gln precipitated less than 3%. Upon immunoelectrophoresis, HDL2 gave a single zone of precipitation with both anti-R-Thr and anti-R-Gln. HDL3 gave two zones of precipitation with anti-R-Thr but gave only one zone with anti-R-Gln. VHDL and GVHDL gave only a single zone of precipitation with anti-R-Thr and no reaction with anti-R-Gln. We conclude: (1) HDL2 and HDL3 are composed of two populations: 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, and (2) VHDL and GVHDL contain R-Thr but do not contain R-Gln.

Human high density lipoproteins (HDL)[1] contain numerous chemically distinct polypeptides (Shore and Shore, 1968, 1969; Scanu *et al.*, 1969; Borut and Aladjem, 1971). The two major polypeptides of apo-HDL are R-Thr and R-Gln, so designated by their respective COOH-terminal amino acids. These polypeptides constitute approximately 85–90% of the protein (Shore and Shore, 1968, 1969; Scanu *et al.*, 1969). In view of the demonstration of the multiplicity of polypeptides in HDL, we were led to ask whether different polypeptides are present in the same or different lipoprotein molecules, and furthermore, what is the fraction of HDL molecules containing a given polypeptide. Both of these questions are crucial to the understanding of lipoprotein structure and function.

In this study we determined the presence of R-Thr and R-Gln polypeptides in HDL2, HDL3, VHDL, and GVHDL from HDL2 and HDL3 by immunoelectrophoresis and we quantitated R-Thr and R-Gln in these lipoproteins by quantitative precipitation using polypeptide specific anti-R-Thr and anti-R-Gln antisera. The results indicate that HDL is composed of at least two populations: one, approximately 90%, which contains both R-Thr and R-Gln, and the other, approximately 10%, which contains R-Thr but not R-Gln. VHDL and GVHDL were found to belong to this latter class, having R-Thr but not R-Gln.

Materials and Methods

Isolation of Antigens. Lipoproteins were isolated from pooled human sera (pools 1 and 2), and from the sera of two individuals (J. J. A. and L. B. C.) who had fasted overnight. The following procedure was used. Serum was brought to a density of 1.063 g/cm^3 with solid NaCl. Centrifugation was carried out in a L2-65B ultracentrifuge for 24 hr at 54,000 rpm (207,400*g*) at 15° using a Spinco 60 Ti rotor. The top 15 ml was removed with a tube slicer. The bottom 20 ml was brought to a density of 1.125 g/cm^3 with solid NaBr, diluted 1:3 with NaCl–NaBr solution of density 1.125 g/cm^3, and centrifuged for 36 hr at 54,000 rpm. The top milliliters containing HDL2 were removed. The bottom 25 ml were adjusted to a density of 1.21 g/cm^3 with solid NaBr, diluted 1:3 with NaCl–NaBr solution of density 1.21 g/cm^3, and centrifuged for 36 hr at 54,000 rpm. The top 10 ml containing HDL3 was then removed with the use of the tube slicer. The bottom 25 ml obtained after the removal of HDL3 was diluted 1:1 with NaCl–NaBr solution of density 1.21 g/cm^3 and spun again at 54,000 rpm for 24 hr. The top 15 ml was removed and the bottom fraction was retained for immunochemical analysis. The lipoproteins remaining in this fraction of density >1.21 g/cm^3 are VHDL. GVHDL was generated from HDL2 or HDL3 by recentrifugation of HDL2 or HDL3 in a NaCl–NaBr solution of density 1.21 g/cm^3 for 48 hr at 54,000 rpm (207,400*g*). The top 30 ml of each tube was removed with the

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[1] Abbreviations used are: HDL, high density lipoprotein; apo-HDL, protein moiety of HDL obtained by extraction with ethanol–ether; HDL2, high density lipoprotein of density 1.063–1.125 g/cm^3; HDL3, high density lipoprotein of density 1.125–1.21 g/cm^3; VHDL, very high density lipoprotein of density >1.21 g/cm^3; GVHDL, very high density lipoprotein generated from HDL2 or HDL3 by recentrifugation in a salt solution of density 1.21 g/cm^3; R-Thr, the major polypeptide of HDL with COOH-terminal threonine; R-Gln, the other major polypeptide of HDL with COOH-terminal glutamine; III and IV refer to two of the major fractions separated from apo-HDL by Sephadex G-200 chromatography. Antisera against HDL2, HDL3, R-Thr, R-Gln, and rabbit γ-globulin will be designated anti-HDL2, etc., respectively.

use of a tube slicer. The bottom 5 ml fraction contained GVHDL.

Two methods were used for the preparation of HDL polypeptides. (1) Apo-HDL2 and apo-HDL3 were prepared by extraction of HDL2 or HDL3 with 3:2 ethanol–ether at −10° (Scanu, 1966); these apoproteins were fractionated by Sephadex G-200 chromatography in 8 M urea (Scanu *et al.*, 1969); (2) apo-HDL2 and apo-HDL3 were delipidated according to the method of Shore and Shore (1967); the polypeptides R-Thr and R-Gln were isolated by DEAE-cellulose chromatography (Shore and Shore, 1968, 1969) as described (Borut and Aladjem, 1971). R-Gln was found to be identical with the R-Gln polypeptide of Shore and Shore by the following criteria: elution from the DEAE-cellulose column between 0.030 and 0.035 M Tris buffer (pH 8.0)–8 M urea, polyacrylamide gel pattern, and amino acid composition (Borut and Aladjem, 1971). The amino acid analysis of the R-Gln fraction was essentially the same as Shore and Shore (1968, 1969) and contained no histidine or arginine as is characteristic of the R-Gln polypeptide. R-Thr was found to be identical with R_1-Thr of apo-HDL2 of Shore and Shore (1969) and the R-Thr of apo-HDL3 of Shore and Shore (1968) by the following criteria: elution from the DEAE-cellulose column between 0.045 and 0.050 M Tris buffer (pH 8.0)–8 M urea, polyacrylamide gel pattern, and amino acid composition (Borut and Aladjem, 1971). The amino acid analysis of the R-Thr fraction was essentially the same as that of Shore and Shore (1968, 1969). This material contained no cysteine or cystine and negligible amounts of isoleucine, as is characteristic of R-Thr (Albers *et al.*, 1971). The polypeptides R-Ala and R-Glu, so designated from their COOH-terminal amino acids, were isolated from VLDL by preparative isoelectric focusing as described by Albers and Scanu (1971).

Prior to iodination, all preparations were dialyzed against four changes of 0.5 M sodium glycine buffer (pH 8.65), containing 10^{-4} M EDTA. Prior to immunochemical analysis, all preparations were dialyzed against 0.05 M Tris-HCl buffer (pH 8.4) containing 10^{-3} M EDTA.

Iodination of Antigens. The lipoprotein and apoprotein preparations were iodinated with ^{125}I by the method of McFarlane (1958), modified as follows. Carrier-free [^{125}I]NaI in 0.1 N NaOH, free from preservative and reducing agents, was obtained from New England Nuclear Corp. The required quantity of [^{125}I]NaI to give a specific activity of approximately 6000 cpm/μg of protein was added to 1 ml of 0.5 M sodium glycine buffer (pH 8.65)–0.5 M NaCl. To this was added the quantity of ICl which would give approximately 2 atoms (average) of iodine/mole of protein. (The ICl solution was freshly prepared by dissolving 0.1 ml of ICl (Eastman) in 660 ml of 0.1 N HCl to give a final concentration of 0.5 mg of ICl/ml.) The iodination mixture ([^{125}I]NaI, ICl, and sodium glycine buffer) was then immediately jetted into the buffered protein solution (0.5 M sodium glycine, pH 8.65) with vigorous stirring. Samples were then dialyzed at 4° for 4 days against 15 changes of 0.05 M Tris-HCl (pH 8.4)–0.03 M NaI–10^{-3} M EDTA, and then against several changes of 0.05 M Tris buffer (pH 8.4) without KI. In each case, more than 98% of the radioactivity in the dialyzed preparation was precipitated by trichloroacetic acid added to a final concentration of 5%. (The labeled lipoproteins had specific activity of 4000–8500 cpm/μg.)

Antisera. The anti-R-Thr, anti-R-Gln, anti-HDL2, anti-HDL3, and goat anti-rabbit γ-globulin antisera were the same as those used in another study (Borut and Aladjem, 1971). These antisera did not react with human serum low density lipoproteins (density 1.035–1.040 g/cm^3), with human serum albumin (Pentex), or with the polypeptides R-Ala and R-Glu isolated from VLDL.

Quantitative Immunochemical Analysis. To determine the fraction of HDL molecules with a particular polypeptide composition, it was first necessary to perform quantitative immunochemical analysis of the isolated polypeptides. The method and calculations used are similar to those used previously (Albers and Dray, 1969b). The method involves precipitation of labeled lipoprotein or polypeptide with specific antibody against a different polypeptide, and/or by goat anti-rabbit γ-globulin to precipitate soluble antigen–antibody complexes. For example, to 10 μg of [^{125}I]R-Thr was added 0.3 ml of anti-R-Thr antiserum, incubated at 37° for 30 min, and then for 48 hr at 4°. The tubes were then centrifuged, and the supernatant fluid was removed and carefully weighed. The precipitate was washed four times with 0.05 M Tris-HCl buffer (pH 8.4) at 4° and then dissolved in 0.5 ml of 0.05 N NaOH. The radioactivity of the supernatant fluid and of the dissolved precipitate was measured in a well-type gamma scintillation counter. The background count was less than 30 cpm. Counting was done for 100 min or 25,000 counts, whichever came first, though some samples were counted for longer periods. The radioactivity of the total supernatant fluid was calculated from the radioactivity of the weighed portion of the supernatant fluid, knowing the total volume and assuming that the contribution of the precipitate to the volume was negligible. All experiments were carried out with 0.3 ml of anti-polypeptide antiserum. This constituted an excess of antibody. The fraction of radioactivity precipitated in the region of antibody excess was independent of antigen concentration over the range of antigen concentration used.

To ensure complete precipitation of the labeled protein, *i.e.*, to precipitate any soluble complexes which might occur, goat anti-rabbit γ-globulin was added to the supernatants, as follows. The supernatant fluid (10 μl) from the initial reaction mixture was added to 0.5 ml of goat anti-rabbit γ-globulin. Previous experiments with labeled γ-globulin and anti-γ-globulin had shown that 0.5 ml represented an excess of antibody. This mixture was incubated for 30 min at 37°, and then at 4° overnight. The tubes were then centrifuged and the radioactivity in the supernatant fluid and precipitate was determined as described above. As controls, labeled R-Thr, R-Gln, HDL2, HDL3, and GVHDL were individually mixed with normal rabbit serum and 10 μl of each of the mixtures were precipitated with goat anti-rabbit γ-globulin.

To determine the extent of nonspecific coprecipitation of R-Thr with R-Gln, and of R-Gln with R-Thr, unlabeled R-Gln was added to an excess of anti-R-Gln in the presence of labeled R-Thr; also, unlabeled R-Thr was added to an excess of anti-R-Thr in the presence of labeled R-Gln. In all control experiments for nonspecific coprecipitation, less than 4% of the radioactivity was found in the precipitate.

To determine the extent of labeling of R-Thr and R-Gln with ^{125}I in HDL, equal quantities (weight) of R-Thr and R-Gln were mixed and then labeled. After dialysis, anti-R-Thr and/or anti-R-Gln were added to aliquots of the mixture. The resulting precipitates and supernatant fluids were treated as stated above, and counted.

The labeled lipoproteins HDL2, HDL3, GVHDL (HDL2), and GVHDL (HDL3) were precipitated with anti-R-Thr and anti-R-Gln in a manner identical with that described above for labeled R-Thr and R-Gln.

[^{125}I]HDL2 and [^{125}I]HDL3 were also precipitated with rabbit anti-HDL2 and rabbit anti-HDL3 antisera, respec-

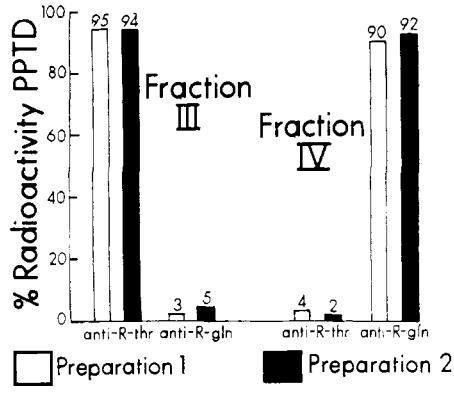

FIGURE 1: Precipitation of fraction [^{125}I]III and fraction [^{125}I]IV with anti-R-Thr and anti-R-Gln. III and IV were obtained from Sephadex G-200 chromatography of apo-HDL2.

tively, in a manner identical with that described above. Again, preliminary experiments were performed to ensure that reactions were carried out in the region of antibody excess.

Immunodiffusion and Immunoelectrophoresis. Immunodiffusion was carried out in 1% agarose–0.05 M Tris-HCl buffer (Albers and Dray, 1968, 1969a). Immunoelectrophoresis was carried out in 1% agarose–barbital buffer (pH 8.4, ionic strength 0.05) (Albers and Dray, 1968, 1969a). The protein concentration of the isolated lipoproteins was approximately 4 mg/ml.

Polyacrylamide gel electrophoresis was carried out at pH 8.9 in 8 M urea by the method of Davis (1964). Acrylamide monomer concentration was 7.5%. The protein was fixed with 10% trichloroacetic acid and stained with Amido Schwarz in 7% acetic acid.

Protein Determination. Protein concentrations were determined by the method of Lowry *et al.* (1951); human serum albumin (Pentex) was used as standard.

Results

Precipitation of Polypeptides. It was first necessary to establish the specificity of the antisera by the method of precipitation of labeled antigen with antisera. The results of a typical experiment using labeled polypeptides isolated by DEAE chromatography and anti-R-Thr and anti-R-Gln antisera are shown in Table I. Anti-R-Thr precipitated 95% of the radioactivity of R-Thr and anti-R-Gln precipitated 96% of the radioactivity of R-Gln. Cross-reactions were less than 3%. Indirect precipitation, that is, addition of 10-μl aliquots of the supernatant to 0.5 ml of goat anti-rabbit γ-globulin, precipitated less than 3% of the supernatant radioactivity. The findings that cross-reactivity between R-Thr and R-Gln preparations is negligible indicate that R-Thr and R-Gln are immunochemically distinct. No differences were observed between polypeptide preparations obtained from apo-HDL2 or apo-HDL3. Also, no differences were observed between different antisera whether the immunizing antigens were obtained from apo-HDL2 or from apo-HDL3.

The calculation of precipitable [^{125}I]HDL from the radioactivity in the precipitates requires a determination of the extent of labeling of the two polypeptides R-Thr and R-Gln per unit weight of protein. When equal quantities (weight) of the two polypeptides were mixed and then labeled, 74% of the radioactivity was precipitated with anti-R-Gln, 34% with anti-R-Thr, and 96% was precipitated with a mixture of anti-R-Thr and anti-R-Gln. Thus, R-Gln is labeled to approximately twice the extent of R-Thr per unit weight of protein.

TABLE I: Precipitation of Labeled R-Thr and R-Gln with Anti-R-Thr and Anti-R-Gln.

Reaction Mixture	Cpm[a] Precipitate	Cpm[a] Supernatant[b]	% of Radioactivity Pptd
(A) 10 μg of [^{125}I]R-Thr	68,529	3,167	95.6
0.3 ml of anti-R-Thr	66,460	3,572	94.9
(B) 10 μg of [^{125}I]R-Thr	1,909	66,291	2.8
0.3 ml of anti-R-Gln	2,094	67,706	3.0
(C) 10 μg of [^{125}I]R-Gln	61,683	2,038	96.8
0.3 ml of anti-R-Gln	58,991	2,459	96.0
(D) 10 μg of [^{125}I]R-Gln	563	61,578	0.9
0.3 ml of anti-R-Thr	1,291	60,143	2.1

[a] Values of duplicate experiments are shown. [b] Corrected for volume loss.

Precipitation of fraction III and fraction IV obtained from Sephadex G-200 chromatography of apo-HDL2 is shown in Figure 1. Anti-R-Thr precipitated 94–95% of the radioactivity of the several ^{125}I-labeled III preparations, whereas anti-R-Gln precipitated only 3–5%. With ^{125}I-labeled IV, anti-R-Thr precipitated between 2 and 4% of the radioactivity, whereas anti-R-Gln precipitated 90–92%. Nearly identical results were obtained when III and IV from apo-HDL3 were similarly precipitated. The fact that anti-R-Thr precipitated about the same percentage of radioactivity from labeled III as from labeled R-Thr, *i.e.*, 95%, indicates that fraction III is composed essentially of R-Thr. Anti-R-Gln precipitated almost the same percentage of radioactivity from labeled IV (91%) as from labeled R-Gln (96%). This indicates that fraction IV is composed essentially of R-Gln.

Gel Diffusion Studies of Isolated Polypeptides. Anti-R-Thr did not react with R-Gln (1–10 mg/ml) in agarose gel, nor did anti-R-Gln react with R-Thr (1–10 mg/ml) as shown previously (Borut and Aladjem, 1971). Anti-R-Thr reacted with R-Thr (1 mg/ml), fraction III (1 mg/ml), and fraction IV (10 mg/ml) to give a line of "identity" among the three antigens. Also, anti-R-Gln reacted with R-Gln (1 mg/ml), fraction III (10 mg/ml), and fraction IV (1 mg/ml) to give a line of "identity" among these three antigens. These findings indicated that III contains immunochemically detectable R-Gln and IV contains immunochemically detectable R-Thr.

Proportion of R-Thr and R-Gln in HDL. As shown in Table II, the relative protein concentrations of the two major peaks obtained by Sephadex G-200 chromatography of apo-HDL2 were found to be 68% for the first peak (fraction III) and 20% for the second peak (fraction IV); 12% of the total protein eluted was in the remaining fractions. This is in agreement with the results of Scanu *et al.* (1969). The weight ratio of R-Thr:R-Gln in HDL2, therefore, is 3.4:1. For HDL3, 74% of the total protein was found in fraction III and 16% in fraction IV. The weight ratio of R-Thr:R-Gln in HDL3 is 4.6:1.

Proportion of Radioactivity in R-Thr and R-Gln in HDL. The results of labeling of the isolated polypeptides may not necessarily reflect the extent of labeling of these polypeptides

TABLE II: Proportion of R-Thr and R-Gln in HDL2 and HDL3.[a]

Sample	Total[b] (mg)	R-Thr[c] (mg)	R-Gln[d] (mg)	R-Thr:Total	R-Gln:Total	R-Thr:R-Gln	R-Thr:(R-Thr + R-Gln)
HDL2	25.5	17.7	4.6	0.69	0.18	3.8	0.79
	24.9	16.5	5.4	0.66	0.22	3.0	0.75
				Av 0.68	0.20	3.4	0.77
HDL3	24.6	18.6	3.8	0.76	0.15	4.8	0.83
	25.1	18.0	4.2	0.72	0.17	4.2	0.81
				Av 0.74	0.16	4.6	0.82

[a] Values obtained from two preparations each are shown. [b] Total protein eluted from column representing approximately 93% of the protein applied. [c] R-Thr is fraction III obtained by Sephadex G-200 chromatography of apo-HDL2 or apo-HDL3. [d] R-Gln is fraction IV obtained by Sephadex G-200 chromatography of apo-HDL2 or apo-HDL3.

in whole HDL. An alternate estimate of the measure of radioactivity in R-Thr and R-Gln in HDL2 and HDL3 was therefore made. HDL2 and HDL3, separately, were labeled with ^{125}I, and were then delipidated. The polypeptides were separated by polyacrylamide gel electrophoresis in 8 M urea (Shore and Shore, 1968). The major band R-Thr and the major band R-Gln were cut out of the gel, and the radioactivity measured. The results of these experiments are shown in Table III. The ratio of radioactivity of R-Thr:R-Gln was found to be 1.6:1 for [^{125}I]HDL2 and 2.1:1 for HDL3. If R-Thr and R-Gln had been equally labeled, then these ratios should have been equal to the weight ratios of R-Thr:R-Gln, *i.e.*, 3.4:1 for HDL2 and 4.6:1 for HDL3 (Table II). The above results indicate that R-Gln is labeled to approximately twice the extent of R-Thr in HDL; for HDL2 2.1:1, *i.e.*, 3.4:1.6; and for HDL3 2.2:1, *i.e.*, 4.6:2.1. This result is consistent with that obtained with the labeling of the isolated polypeptides. Furthermore, the relative extent of labeling of these two polypeptides per weight of protein agrees well with the relative amounts of tyrosine available for iodination; *i.e.*, 45 moles of tyrosine/10^3 moles of amino acids for R-Gln and 28 moles of tyrosine/10^3 moles of amino acids for R-Thr (Shore and Shore, 1968).

TABLE III: Radioactivity in R-Thr and R-Gln of Labeled HDL2 and HDL3.[a]

Sample	R-Thr[b] (cpm)	R-Gln[c] (cpm)	R-Thr:R-Gln	R-Thr:(R-Thr + R-Gln)
HDL2	65,282	40,182	1.62	0.619
	85,827	57,939	1.48	0.597
			Av 1.55	0.608
HDL3	63,940	31,811	2.01	0.668
	119,886	55,247	2.17	0.684
			Av 2.09	0.676

[a] Each value shown is an average of four to six replicate analyses. Two HDL2 and two HDL3 preparations were analyzed. [b] R-Thr is the first major band obtained by polyacrylamide gel electrophoresis of [^{125}I]apo-HDL2 or [^{125}I]-apo-HDL3. [c] R-Gln is the second major band obtained by polyacrylamide gel electrophoresis of [^{125}I]apo-HDL2 or [^{125}I]apo-HDL3.

Subpopulations of HDL with Different Polypeptide Compositions. Figure 2 shows the results of immunoelectrophoresis of human serum and HDL2 reacting with anti-R-Thr and anti-R-Gln antisera. Single zones of precipitation were obtained. Figure 3 shows the results of immunoelectrophoresis of HDL3. HDL3 reacting with anti-R-Thr gave two zones of precipitation with different mobilities. HDL3 reacting with anti-R-Gln gave only a single zone of precipitation with mobility corresponding to the faster zone of HDL3 reacting with anti-R-Thr.

The results of quantitative precipitation of [^{125}I]HDL with specific antisera are given in Tables IV, V, and VI. The following are the results: (1) 93–97% of the radioactivity was precipitated by (i) anti-HDL2 or anti-HDL3, followed by goat anti-rabbit γ-globulin, (ii) anti-R-Thr, followed by anti-γ-

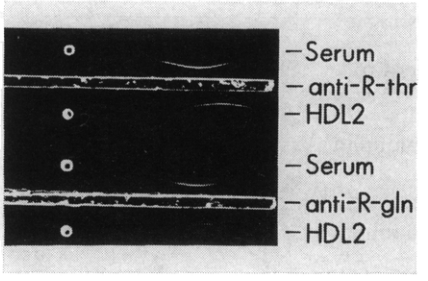

FIGURE 2: Immunoelectrophoresis in 1% agarose showing anti-R-Thr and anti-R-Gln reacting with whole serum and HDL_2.

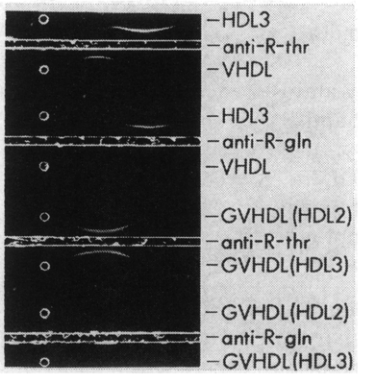

FIGURE 3: Immunoelectrophoresis in 1% agarose showing the precipitin reactions of anti-R-Thr and anti-R-Gln reacting with HDL3, VHDL, and GVHDL (HDL2 or HDL3).

TABLE IV: Precipitation of [^{125}I]HDL2 (Pool 1) with Anti-R-Thr and Anti-R-Gln.[a]

Reaction Mixture	Cpm		% Radioactivity Pptd	Cumulative Radioactivity Ptpd
	Precipitate	Supernatant[b]		
(A) 10 μg of [^{125}I]HDL2 + 0.3 ml of anti-R-Thr	73,233	5,541	93.0	
	74,468	5,468	93.2	
(B) 0.010 ml of supernatant A + 0.5 ml of anti-γ-globulin	74	106	41.1	95.9[c]
	80	96	45.4	96.3
(C) 10 μg of [^{125}I]HDL2 + 0.3 ml of anti-R-Gln	64,480	15,537	80.6	
	64,128	14,274	81.8	
(D) 0.010 ml of supernatant C + 0.5 ml of anti-γ-globulin	157	332	32.1	86.8
	134	346	27.9	86.9
(E) 0.150 ml of supernatant C + 0.3 ml anti-R-Thr + 9 μg of HDL2 unlabeled	4,963	2,780	64.1	93.0
	4,467	2,534	63.8	93.4
(F) 0.010 ml of supernatant E + 0.5 ml of anti-γ-globulin	16	46	25.8	94.8
	18	37	32.7	95.6

[a] Values obtained in duplicate experiments are shown. [b] Corrected for volume loss. [c] The value was obtained as follows: 41.1 (100 − 93.0) = 2.9%; 93.0 + 2.9 = 95.9.

globulin, and (iii) anti-R-Gln, followed by anti-R-Thr, followed by anti-γ-globulin. (2) Only 84–90% of the radioactivity was precipitated by anti-R-Gln followed by anti-γ-globulin. We concluded that HDL is composed of two subpopulations: one which contains both R-Thr and R-Gln, and the other, which contains R-Thr but not R-Gln.

TABLE V: Precipitation of Whole [^{125}I]HDL2 by Antisera.[a]

Antiserum	Pool 1	Pool 2	Serum JA	Serum LC
Anti-HDL2	94[b]	93	95	93
Fb[c] anti-γ-globulin	2	1	1	1
Total A	96	94	96	94
Anti-R-Thr	93	91	94	93
Fb anti-γ-globulin	3	2	1	1
Total B	96	93	95	94
Anti-R-Gln	81	82	88	80
Fb anti-γ-globulin	6	4	2	6
Total C	87	86	90	86
Anti-R-Gln	81	82	88	80
Fb anti-R-Thr	12	10	6	13
Fb anti-γ-globulin	2	2	1	3
Total D	95	94	95	96
Precipitated by anti-R-Thr but not anti-R-Gln[d]	8.5	7.5	5	9
Per cent of HDL2 precipitated by anti-R-Thr but not anti-R-Gln[e]	11	10	6	12

[a] For experimental procedures, see Methods. [b] Per cent of total radioactivity precipitated; each value represents the average of duplicate experiments. [c] Fb = followed by. [d] By difference = [(total B + total D)/2] − total C. [e] For calculations, see Results.

The next to last columns in Tables V and VI show that between 5 and 9.5% of the total radioactivity of the various preparations were precipitable with anti-R-Thr but were not precipitable with anti-R-Gln. To calculate the fraction of the HDL population which does not contain R-Gln but does contain R-Thr, we assume that the extent of labeling of R-Thr is the same in all molecules containing R-Thr, and that both subpopulations have the same quantity of protein per molecule. Then, the fraction of HDL which does not contain R-Gln but does contain R-Thr is shown in eq 1. This fraction × 100

TABLE VI: Precipitation of Whole [^{125}I]HDL3 by Antisera.[a]

Antiserum	Pool 1	Pool 2	Serum JA	Serum LC
Anti-HDL3	94[b]	93	94	93
Fb[c] anti-γ-globulin	1	1	2	2
Total A	95	94	95	95
Anti-R-Thr	91	92	95	93
Fb anti-γ-globulin	3	2	1	1
Total B	94	94	96	94
Anti-R-Gln	83	83	88	79
Fb anti-γ-globulin	5	4	2	5
Total C	88	87	90	84
Anti-R-Gln	83	83	88	79
Fb anti-R-Thr	9	9	8	13
Fb anti-γ-globulin	2	1	1	1
Total D	94	93	97	93
Precipitated by anti-R-Thr[d] but not anti-R-Gln	6	6.5	6.5	9.5
Per cent of HDL3[e] precipitated by anti-R-Thr but not anti-R-Gln	8	8	8	12

[a] Footnotes same as Table V.

$$\frac{\text{fraction of radioactivity in HDL with R-Thr but not R-Gln}}{\text{radioactivity ratio R-Thr:(R-Thr + R-Gln)}} \times \frac{\text{R-Thr (mg)}}{\text{(R-Thr + R-Gln) (mg)}} \times 1.05 \quad (1)$$

is given in Tables V and VI for each of the HDL preparations. The radioactivity ratio R-Thr:(R-Thr + R-Gln) is 0.608 from HDL2 and 0.676 for HDL3 as shown in Table II. The value R-Thr (mg)/(R-Thr + R-Gln) (mg) is 0.77 for HDL2 and 0.82 for HDL3 as shown in Table II. The value 1.05 is a minor correction which is applied due to the fact that only approximately 95% but not 100% of the radioactivity was precipitable with anti-HDL2 or anti-HDL3 antisera. For example, the percentage of HDL3 (pool 1) which does not contain R-Gln but does contain R-Thr is equal to $6/0.676 \times 0.82 = 7.3$; $7.3 \times 1.05 = 7.7$. As seen in Tables V and VI, approximately 10% of both HDL2 and HDL3 do not contain R-Gln but do contain R-Thr and approximately 90% contain both R-Thr and R-Gln.

Absence of R-Gln in VHDL and GVHDL. Figure 3 shows the results of immunoelectrophoresis of VHDL, GVHDL (HDL2), and GVHDL (HDL3) reacting with anti-R-Thr and anti-R-Gln. A single zone of precipitation was obtained with anti-R-Thr, corresponding to the slower zone of HDL3 reacting with anti-R-Thr. No reaction was obtained with anti-R-Gln.

The percentage of radioactivity precipitated with anti-R-Thr and anti-R-Gln from GVHDL is shown graphically in Figure 4. Anti-R-Gln precipitated 1–3% while anti-R-Thr precipitated 96–97% of the radioactivity in GVHDL from HDL2 and HDL3. We conclude that all GVHDL molecules, whether from HDL2 or from HDL3, do not contain R-Gln but do contain R-Thr.

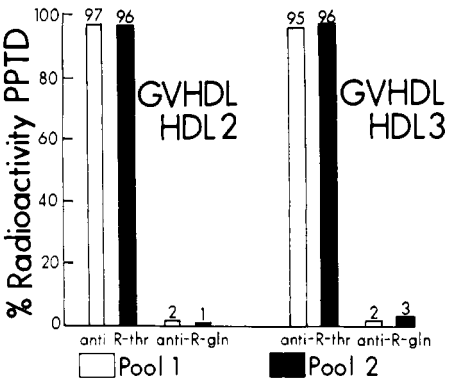

FIGURE 4: Precipitation of [^{125}I]GVHDL with anti-R-Thr and anti-R-Gln. GVHDL was obtained from HDL2 or HDL3 by centrifugation.

Discussion

Precipitation of labeled lipoproteins with specific anti-polypeptide antisera represents a new approach to the study of lipoprotein structure. By this method the presence of a given polypeptide in a heterogeneous population of molecules can be ascertained and quantitated. In the present study, antisera against the two major polypeptides of HDL, R-Thr, and R-Gln were used. The major findings are: (1) HDL is composed of two populations. One population contains both R-Gln and R-Thr; the other population does not contain R-Gln but does contain R-Thr. (2) VHDL and GVHDL do not contain R-Gln but do contain R-Thr.

Using immunodiffusion and immunoelectrophoresis, Borut and Aladjem (1971) have reported the presence in HDL of molecules which contain R-Thr but not R-Gln. The present results of precipitation of labeled HDL2 yield quantitative estimates of the concentrations of these populations. Eighty-eight to ninety-four per cent of HDL2 contain both R-Gln and R-Thr; six to twelve per cent do not contain R-Gln, but do contain R-Thr.

Nearly identical results were obtained by precipitation of labeled HDL3. Eighty-eight to ninety-two per cent of HDL3 contain both R-Gln and R-Thr; eight to twelve per cent do not contain R-Gln, but do contain R-Thr.

The present study shows that VHDL and GVHDL do not contain R-Gln but do contain R-Thr. Levy and Frederickson (1965) reported the presence of two components of HDL by immunoelectrophoresis using anti-HDL antisera. The faster migrating component was observed in whole plasma, HDL2, and HDL3 and was called LpA. The slower migrating component was found in HDL3 and the 1.21 infranatant, and was called LpB. Borut and Aladjem (1971) reported the presence of two major components and several minor components of HDL by immunoelectrophoresis. HDL2 reacting with anti-R-Thr and anti-R-Gln antisera yielded one major precipitin line analogous to LpA. HDL3 reacting with anti-R-Thr antisera gave two major zones of precipitation analogous to LpA and LpB. The present studies show that VHDL and GVHDL have electrophoretic mobility similar to LpB and, like LpB, do not contain R-Gln but do contain R-Thr. Since molecules with R-Thr but not R-Gln can be generated by centrifugation, it is possible that the R-Gln-less subpopulation may have been produced as a result of centrifugation.

The present results of precipitation of labeled GVHDL prepared from HDL2 or HDL3 show that all GVHDL molecules contain R-Thr and that none contain R-Gln. Scanu and Granda (1966) generated VHDL by recentrifugation of HDL2 or HDL3 at 1.21 g/cm^3. The top milliliter (HDL2 or HDL3) and bottom milliliter (GVHDL) were found to be identical in amino acid and carbohydrate compositions. They concluded that the differences between the top and bottom fractions were due to differences in the lipid moiety. More recently, centrifugation of sonicated or ether extracted HDL2 yielded a fraction of density >1.21 g/cm^3 (VHDL) which was reported to be similar to fraction III (Scanu *et al.*, 1970).

The results of quantitative precipitation of labeled fraction III or IV obtained by Sephadex G-200 chromatography show that fraction III is composed essentially of R-Thr and that fraction IV is composed essentially of R-Gln. Comparison of the amino acid data of Shore and Shore (1968, 1969), and to that of Scanu *et al.* (1969), indicates that the polypeptides R-Thr and R-Gln closely resemble III and IV, respectively, thus corroborating the immunochemical evidence that III and IV are composed essentially of R-Thr and R-Gln, respectively. It should be noted that our gel diffusion studies indicate that III does contain a small amount of R-Gln and that IV contains R-Thr. This result is consistent with the chemical evidence and explains why trace amounts of half-cystine and 1.8 moles of isoleucine/100,000 g of protein were found in III, and why 1.6 moles of histidine and 1.0 mole of arginine/100,000 g of protein were found in fraction IV (Scanu *et al.*, 1969). R-Gln isolated from apo-HDL2 or apo-HDL3 by DEAE chromatography has been shown not to contain histidine or arginine. R-Thr isolated from HDL3 contains no half-cystine or isoleucine. R-Thr from HDL2, however, was

reported to contain variable amounts of isoleucine, *i.e.*, 0–1 mole/mole of polypeptide (Shore and Shore, 1968, 1969).

It has recently been shown by preparative isoelectric focusing in a narrow pH gradient that III from apo-HDL2 contains five isoelectrically distinct subfractions with nearly identical amino acid compositions (Albers *et al.*, 1971). The composition of these fractions is similar to that previously published for R-Thr (Shore and Shore, 1968, 1969). All five fractions contained detectable amounts of isoleucine with a maximum of 0.3 mole of isoleucine/mole of polypeptide. A sixth fraction was also found which was heterogeneous with respect to isoelectric point and represented approximately 5% of the total protein. This fraction had an amino acid composition similar to R-Gln.

The present finding that both HDL2 and HDL3 contain subpopulations with differing polypeptide compositions may be important because subpopulations with differing polypeptide compositions are probably a general occurrence in all lipoprotein hydrated density classes. In the present study, only antisera against the two major polypeptides were used. Since HDL contains several additional polypeptides (Shore and Shore, 1968, 1969; Scanu *et al.*, 1969; Borut and Aladjem, 1971), it is reasonable to suspect that subpopulations with respect to the presence or absence of these polypeptides may also occur. We know that some of the HDL polypeptides occur in VLDL and chylomicrons, while at least one does not (Pearlstein *et al.*, 1971). Furthermore, anti-HDL antisera have been found by qualitative precipitin analyses to precipitate a fraction of any given VLDL and chylomicron preparation but they do not precipitate all these lipoproteins from a given serum (to be published). This suggests that VLDL and chylomicrons also contain subpopulations with differing polypeptide composition. This is evidence, therefore, that polypeptide composition heterogeneity probably is a general phenomenon and that it may occur in every lipoprotein hydrated density class. The physiologic and metabolic function of these subpopulations must be investigated. A classification of lipoproteins in terms of their polypeptide composition might be appropriate.

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