Identification and characterization of rat serum lipoprotein subclasses. Isolation by chromatography on agarose columns and sequential immunoprecipitation

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Abstract Lipoproteins, present in serum of chow-fed rats, were fractionated according to size by chromatography of serum on 6% agarose columns. The distributions of apolipoprotein (apo) A-I, E, and A-IV within the high density lipoprotein (HDL) size range (i.e., lipoprotein complexes smaller than low density lipoproteins) showed the existence of lipoprotein subclasses with different size and chemical composition. Sequential immunoprecipitations were performed on these fractions obtained by agarose column chromatography, using specific antisera against apoA-I, apoE, and apoA-IV. The resulting precipitates and supernatants were analyzed for cholesteryl esters, unesterified cholesterol, phospholipids, triglycerides, and specific lipoproteins. The following conclusions were drawn from these experiments. *1*) Sixty-three ± 3% of apoE in the total HDL size range is present on a large particle (mol wt 750,000). This lipoprotein contains apoE as its sole protein constituent and is called LpE. Thirty-nine ± 4% of the cholesterol found in the HDL size range is present in this fraction. The cholesterol:phospholipid ratio is 1:1.1. *2*) Sixty-nine ± 8% of apoA-I in the total HDL size range is present on a smaller particle (mol wt 250,000). This apoA-I-HDL has apoA-I as its major protein component and possibly contains minor amounts of C apoproteins and A-II, but neither apoE nor apoA-IV. It contains 39 ± 8% of the total cholesterol found in the HDL size range and the cholesterol:phospholipid ratio is 1:1.6. *3*) Twenty-one ± 8% of apoA-IV in the total HDL size range is present on a particle that does not contain any apoE or apoA-I, and only 4 ± 1% of the total cholesterol present in the HDL size range. *4*) The remainder of the cholesterol found in the HDL size range is mostly present on two complex lipoproteins: HDL containing both apoA-I and apoA-IV and HDL containing both apoA-I and apoE.—**Dallinga-Thie, G. M., V. L. M. Schneijderberg, and A. van Tol.** Identification and characterization of rat serum lipoprotein subclasses. Isolation by chromatography on agarose columns and sequential immunoprecipitation. *J. Lipid Res.* 1986. **27:** 1035–1043.

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Classification of different serum lipoprotein classes according to their apolipoprotein composition has been described by Alaupovic (1). A distinction was made between so-called simple lipoproteins, containing only one apolipoprotein (apo), and complex lipoproteins, containing more than one apolipoprotein. According to this classification, human HDL can be divided into one simple lipoprotein (Lp A-I), containing only apoA-I, and a complex lipoprotein particle Lp (A-I with A-II), containing both apoA-I and apoA-II (1–5).

Rat HDL subclasses have been described by Weisgraber, Mahley, and Assmann (6). Using ultracentrifugation and Geon-Pevikon block electrophoresis, they isolated an HDL fraction (HDL_1), with apoE as its major apolipoprotein, from normal rat serum and a similar HDL fraction (HDL_c) from serum of cholesterol-fed rats. These fractions, however, still contain small amounts of apoA-I. Rat HDL subfractions were also separated by heparin-Sepharose column chromatography (7). Two HDL subfractions can be obtained using this method: HDL enriched in apoE and HDL enriched in apoA-I.

Evidence for the existence of different HDL subfractions also comes from experiments using agarose column chromatography. When rat serum lipoproteins were separated according to size it was found that the elution profiles of apoE and apoA-I were not identical, as apoE was eluted ahead of apoA-I in the HDL size range (8, 9). This suggests that apoE is enriched on the larger HDL particles. In order to analyze the different apoA-IV-containing fractions in the HDL size range, we recently used specific im-

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; LpE, lipoprotein E; EDTA, ethylenediamine tetraacetic acid; IgG, γ-immunoglobulins; TCA, trichloroacetic acid; DOC, deoxycholate.

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munoprecipitations (9). This technique provided evidence for the existence of three types of HDL particles of relatively small size: HDL containing apoA-IV, HDL containing apoA-I, and a more complex HDL particle, containing both apoA-I and apoA-IV; none of these three subclasses contained appreciable amounts of apoE. We now report the identification and chemical composition of five distinct rat lipoproteins, present in the HDL size range, isolated from rat serum using 6% agarose column chromatography and characterized by sequential immunoprecipitation with specific antibodies against apoA-I, apoE, and apoA-IV. A major finding is that almost 40% of the cholesterol found in the HDL size range in serum from chow-fed rats is carried by a lipoprotein containing apoE as its sole protein constituent.

MATERIALS AND METHODS

Isolation of serum HDL

Male Wistar rats, weighing 300–350 g, were maintained on standard laboratory chow and tap water. Blood was collected at 9 AM from fed animals by aortic puncture under light ether anesthesia. The blood was kept on ice for 2 hr. Serum was obtained by low speed centrifugation at 4°C and EDTA solution (pH 7.4) was added to a final concentration of 1 mM. Ten ml of pooled serum from two rats, containing 10% sucrose (w/v), was applied on a 6% agarose column (2 × 120 cm, Bio-Rad, Richmond, CA), equilibrated, and eluted with 0.15 M NaCl containing 2 mM Na-phosphate buffer (pH 7.4), 0.01% NaN_3, and 1 mM EDTA (9). The column was operated at 4°C. Compared to a previous publication (9), we changed the column size and the flow rate; the columns had a diameter of 2 cm, compared to the 2.5-cm diameter columns used previously. The flow rate was 10 ml/hr (previously 15 ml/hr). The length of the column was kept at 120 cm. These changes clearly resulted in an improved separation of the lipoprotein classes present in rat serum.

Standardization of the columns

Several markers were used in order to standardize the agarose columns. Iodinated rat serum albumin, rat serum VLDL and HDL, human LDL, HDL_2, and HDL_3 were used for calibration (as shown later in Fig. 2). These lipoproteins were isolated by ultracentrifugation in salt solutions of the appropriate densities. In order to estimate the size of the different lipoprotein fractions, the column was also standardized using a gel filtration standard kit (Bio-Rad) containing proteins with known molecular weights, i.e., thyroglobulin (mol wt 670,000), γ-globulin (mol wt 158,000), ovalbumin (mol wt 44,000), myoglobulin (mol wt 17,000), and vitamin B-12 (mol wt 1,350). When the logarithms of these molecular weights were plotted against V_e/V_o (elution volume/void volume), a linear relationship was found.

Immunoassays

ApoA-I, apoA-IV, and apoE concentrations were determined by electroimmunoassay, exactly as described previously (10, 11). Briefly, proteins present in sera, immunoprecipitates, supernatants, column fractions, and standards were routinely precipitated with TCA in the presence of DOC as a carrier. The resulting protein pellets were solubilized in 0.1 ml of 0.5 M NaOH and delipidated with tetramethyl urea. Twenty mM Tris–HCl (pH 8.3), containing 8 M urea, was added before the samples were analyzed by immunoelectrophoresis (11).

Quantitative immunoprecipitation

Rabbit anti-apoA-I, anti-apoA-IV, and anti-apoE IgG, prepared as described (11), were used for the quantitative immunoprecipitation of apoA-I, apoE-, and apoA-IV-containing lipoproteins. The "HDL" fraction obtained from rat serum by agarose column chromatography was divided into two pools. Pool A, with fractions containing apoE as their major protein constituent, eluted between 205 and 235 ml and pool B, with fractions containing apoA-I and apoA-IV as major apoprotein components, eluted between 240 and 274 ml (see Fig. 1.). Two-step immunoprecipitation studies were carried out. In the first step the lipoproteins were subjected to precipitation with a combination of two different antibodies (for details see Results). The amount of antibody needed for quantitative precipitation of the apoprotein was determined in a separated experiment. Incubations were carried out overnight at 4°C and the immunoprecipitates were collected by low speed centrifugation at 4°C (30 min at 2700 rpm). The resulting supernatant was treated with the third antibody (see Results) and a second precipitate was isolated, as described above. ApoA-I, apoE, and apoA-IV concentrations were measured both in the two precipitates and in the final supernatant. Recoveries, as compared to pool A or pool B, were 99 ± 10%, 99 ± 9%, and 100 ± 9% for apoA-I, apoA-IV, and apoE, respectively. The supernatants and the precipitates were extracted according to Bligh and Dyer (12), followed by determination of cholesterol (esters) and phospholipids in the extract. After evaporation of the chloroform phase under a stream of nitrogen, unesterified cholesterol and total phospholipids (13) were determined. Cholesterol was assayed without further treatment using an enzymatic method (CHOD-PAP kit, Boehringer Mannheim, F.R.G., cat. no. 310328). Cholesteryl esters were saponified with alcoholic KOH and, after extraction with petroleum ether, total cholesterol was determined, using the enzymatic method. Cholesteryl esters were expressed as the calculated difference between total cholesterol and unesterified cholesterol. One hundred one ± 7% of total

cholesterol and 99 ± 4% of the phospholipids present in pool A or pool B were recovered in the two precipitates. ApoA-I, apoA-IV, apoE, cholesterol, and phospholipid were absent from the final supernatant, after correction for small amounts of cholesterol and phospholipid present in the antibody preparations. Triglycerides measured according to Laurell (14) could not be detected in any of the HDL fractions isolated by agarose column chromatography with size smaller than LDL. This is not surprising as it is known that the triglyceride content of total rat HDL, isolated by ultracentrifugation, is only 0.5%. The assay used was not sensitive enough to measure these small amounts in the diluted HDL subfractions obtained by gel filtration.

Aliquots (25 μl) of the eluted HDL subfractions were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels according to Cleveland et al. (15), in order to determine the presence of low molecular weight apolipoproteins (e.g., C apoproteins and apoA-II, see Fig. 3).

RESULTS

Isolation of rat serum lipoproteins by agarose column chromatography

The elution pattern of serum protein and the distribution of cholesterol after gel filtration of rat serum on 6% agarose is presented in **Fig. 1A.** The cholesterol profile of another experiment is shown in **Fig. 2.** All columns were standardized using VLDL, LDL, and HDL fractions isolated by density flotation in the ultracentrifuge. Four cholesterol peaks can be distinguished. The cholesterol eluted in the void volume can be ascribed to chylomicrons plus VLDL. The second small cholesterol peak (sometimes in the form of a shoulder) is eluted in the LDL size range (see Fig. 2). This LDL fraction as well as the lipoproteins present in the void volume were not investigated further. Fig. 1A shows that two additional major cholesterol peaks are eluted between 200 and 280 ml. The peak in absorbance at 280 nm, eluted between 200 and 220 ml, is caused by immunoglobulin M (mol wt 900,000). The elution patterns of apoA-I and apoE are shown in Fig. 1B and Fig. 2A and B. Some apoE and very small amounts of apoA-I are present in the void volume (chylomicrons plus VLDL), but the bulk of both apolipoproteins is found in the HDL size range (lipoprotein complexes smaller than LDL). The elution profiles of apoA-I and apoE in the HDL region were clearly nonidentical. Figs. 1B and 2B show that a major fraction of the apoE coelutes with the third cholesterol peak, indicating the presence of apoE on relatively large particles. ApoA-I coelutes with the fourth cholesterol peak representing HDL particles with a relatively small size. The elution profile of apoA-IV is different from that of apoA-I (9, 16, 17, and Fig. 2C). As reported before (9), about 65% of the apoA-IV present in serum of chow-fed rats coelutes with apoA-I, while 35% is present as "free" apoA-IV that elutes from the column clearly after HDL cholesterol and HDL apoA-I. Fig. 2C shows that "free" apoA-IV is eluted from the 6% agarose columns after rat serum albumin (RSA). The distributions of apoA-I and apoE, shown in Fig. 1B and in Fig. 2A and B, already provide strong evidence in favor of the existence of various lipoprotein subclasses in the HDL size range in normal rat serum.

Identification and characterization of lipoprotein subclasses by sequential immunoprecipitation

Lipoproteins, isolated by molecular sieve chromatography on 6% agarose, were divided into two pools based on their apolipoprotein composition: pool A, eluted between 205–235 ml, containing the bulk of the apoE, and pool B, eluting between 240–274 ml, containing the bulk of apoA-I and apoA-IV. Both pools were subjected to a series of immunoprecipitations, using two antibodies in the first step and one antibody in the second precipitation step. When all fractions containing the lipoproteins within the HDL size range were pooled together (fractions eluted between 205 and 274 ml) and subsequently analyzed by sequential immunoprecipitation, all five lipoprotein subclasses described below could be identified (results not shown). Pool A and pool B were used for practical reasons only.

In order to determine the "molecular weight" of lipoprotein particles, the column was calibrated with a mixture of pure protein markers with known molecular weights. A calibration curve can be constructed by plotting elution volume/void volume versus the logarithm of molecular weight. The "molecular weights" of the lipoproteins carrying the bulk of the cholesterol, present in pool A or pool B, can be roughly estimated using this standard curve. LpE and apoA-I-HDL (see below) represent more than 70% of the cholesterol present in pool A and pool B, respectively. However, the determination of the molecular weights of the other HDL subfractions cannot be performed in the same way because they represent only minor fractions of the total cholesterol present in pool B.

When pool A was treated with a combination of antibodies against apoA-I and apoA-IV (**Table 1**), only 18% of the apoE was coprecipitated, together with 100% of the apoA-I and apoA-IV. The resulting supernatant was subsequently treated with anti-apoE IgG (Table 1). This treatment results in the quantitative recovery of apoE in this second precipitate. Because apoE turned out to be the only apolipoprotein present in the lipoprotein complex precipitated by anti-apoE (see below), this lipoprotein was named LpE. LpE has a particle weight of about 750,000. Because of our inability to measure apoC or apoA-II by means of electroimmunoassay, due to a lack of proper antisera, we analyzed all original column fractions as well as the immunoprecipitates on SDS polyacrylamide gel electrophoresis. However, because the immunoprecipitates contain large amounts of IgG in addition to the apolipoproteins, it was not possible to visualize the apolipoproteins present

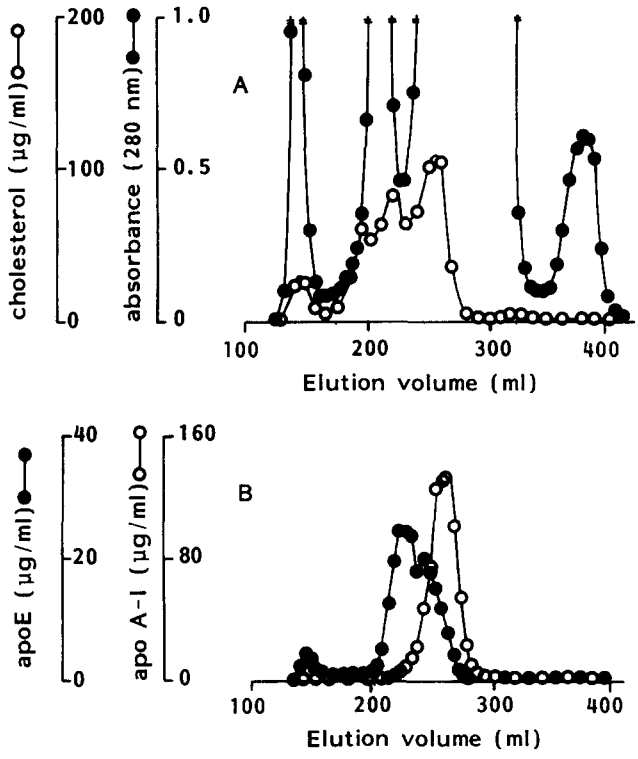

Fig. 1. Separation on a column of 6% agarose gel of serum obtained from chow-fed rats. Ten ml of serum was applied to a column of 6% agarose (2 × 120 cm), equilibrated with 0.15 M NaCl, containing 2 mM Na-phosphate, pH 7.4, 1 mM EDTA, and 0.1% NaN_3, at 4°C. The lipoproteins were eluted with the same medium at a flow rate of 10 ml/hr. Fig. 1A shows the absorbance of serum proteins at 280 nm (●) and the distribution of cholesterol (○) in the eluted fractions. Fig. 1B shows the elution profiles of apoA-I (○) and apoE (●) mass, as determined by electroimmunoassay. This figure shows one representative experiment out of a series of four.

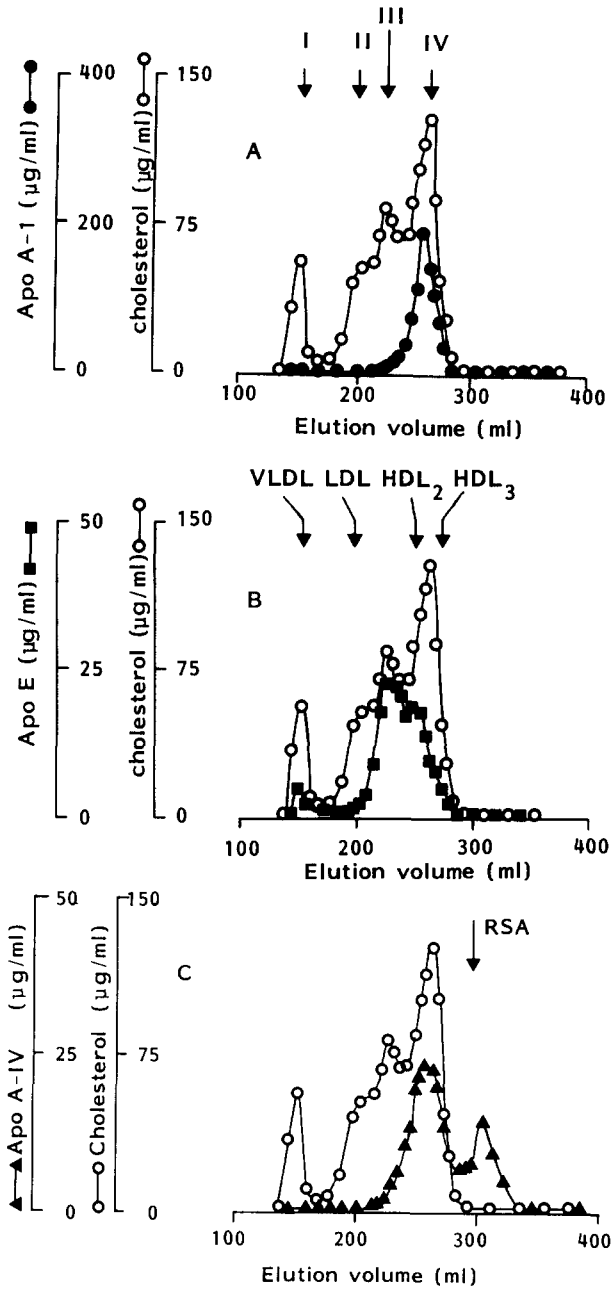

Fig. 2. Serum obtained from chow-fed rats was chromatographed on a 6% agarose column, exactly as described in the legend to Fig. 1. Fig. 2A shows the elution profiles of cholesterol (○) and apoA-I (●). I, II, III, and IV indicate the elution volumes of the different cholesterol-containing fractions, which can be distinguished after chromatography (c.f., Fig. 1). The highest levels of apoA-I are present in fraction IV. Fig. 2B shows the elution profiles of cholesterol (○) and apoE (■). VLDL, LDL, HDL_2 and HDL_3 indicate the elution volumes of isolated human lipoproteins that were chromatographed in separate experiments. VLDL and LDL were isolated by sequential ultracentrifugation (34) and washed once. Human HDL_2 and HDL_3 were isolated by rate zonal density gradient ultracentrifugation in a swinging bucket rotor, as described by Groot et al. (35). Peaks in the elution profile of apoE are found in fraction I (VLDL ≈ chylomicrons present in void volume), in fraction III (LpE, with particle size between that of human LDL and HDL_2), and in HDL particles with a size close to that of human HDL_2. Fig. 2C shows the elution profile of cholesterol (○) and apoA-IV (▲). RSA indicates the elution volume of rat serum albumin.

in the immunoprecipitates by this technique. The gels of the original column fractions shown in **Fig. 3** reveal that pool A, containing the bulk of the apoE, does not contain any apoC or apoA-II, while pool B, containing the bulk of HDL apoA-I and HDL apoA-IV, does contain low molecular weight apolipoproteins. We therefore conclude that LpE is free of apoC and apoA-II. These low molecular weight apolipoproteins may be present on apoA-I-HDL, or apoA-IV-HDL, as well as on any of the more complex HDL subfractions (see below). Specific immunoassays for apoCs and apoA-II are needed to substantiate this point. On the basis of the data shown in Table 1, it is possible to calculate the following chemical composition for LpE: 12% protein, 47% phospholipids, and 41% cholesterol (35% of this cholesterol is in the unesterified form). The protein content is calculated using the amount of apoE determined by quantitative electroimmunoassay.

Another simple lipoprotein complex was isolated from the column fractions containing the bulk of the apoA-I (pool B), by a first precipitation with a combination of anti-apoE and anti-apoA-IV IgGs (**Table 2**). Only 23% of the apoA-

TABLE 1. LpE isolated by agarose column chromatography and immunoprecipitation

Fraction	Original HDL[a] (Pool A)	Fraction Precipitated with Anti-ApoA-IV and Anti-ApoA-I	Fraction Subsequently Precipitated with Anti-ApoE[b]
ApoA-I	9.0 ± 3.1[c]	9.1 ± 3.4	0.8 ± 0.3
ApoA-IV	4.7 ± 0.2	4.5 ± 0.6	0.3 ± 0.5
ApoE	19.8 ± 2.3	3.3 ± 0.4	16.2 ± 0.6
Cholesterol	78.5 ± 17.2	19.1 ± 2.6	57.4 ± 4.7[d]
Phospholipids	81.5 ± 23.5	23.1 ± 8.1	65.4 ± 21.3

[a]Fractions containing the bulk of apoE were pooled (e.g., Fig. 1: 205–235 ml).
[b]ApoA-I, apoA-IV, and apoE were not detectable in the final supernatant.
[c]Results are expressed as μg/ml column fraction. Values are mean ± SD (n = 3).
[d]Of the total cholesterol, 34.9 ± 2.5% was present as unesterified cholesterol.

I was coprecipitated in this step. This indicated that only 23% of the apoA-I is present on complex HDL particles containing also apoE and/or apoA-IV. The remaining 77% of the apoA-I was subsequently precipitated from the supernatant of the first precipitation step with anti-apoA-I IgG. Neither apoE nor apoA-IV could be detected in this second precipitate. We propose to name this simple lipid–protein complex apoA-I-HDL, because it carries apoA-I as its only major apolipoprotein. However, it cannot be excluded that apoC or apoA-II are also present (see Fig. 3). The composition of apoA-I-HDL resembles that of total rat HDL. Its chemical composition is 34% protein, 26% cholesterol (18% of this cholesterol is in the unesterified form), and 40% phospholipids. ApoA-I-HDL is relatively small (particle weight approximately 250,000).

A third simple lipid–protein complex can also be isolated from the column fractions containing the bulk of the apoA-I (pool B). Twenty-one percent of the apoA-IV in this pooled fraction is not precipitated by a combination of anti-apoA-I and anti-apoE IgGs (**Table 3**). Subsequently apoA-IV-HDL can be quantitatively precipitated with anti-apoA-IV. ApoA-IV-HDL is a protein-rich HDL particle. From the data presented in Table 3 it can be calculated that it contains about 48% protein, 22% cholesterol (57% of this cholesterol is in the unesterified form), and 30% phospholipids. We can conclude that the remaining 79% of the apoA-IV in pool B is present on a lipoprotein particle together with apoA-I inasmuch as the results of earlier ex-

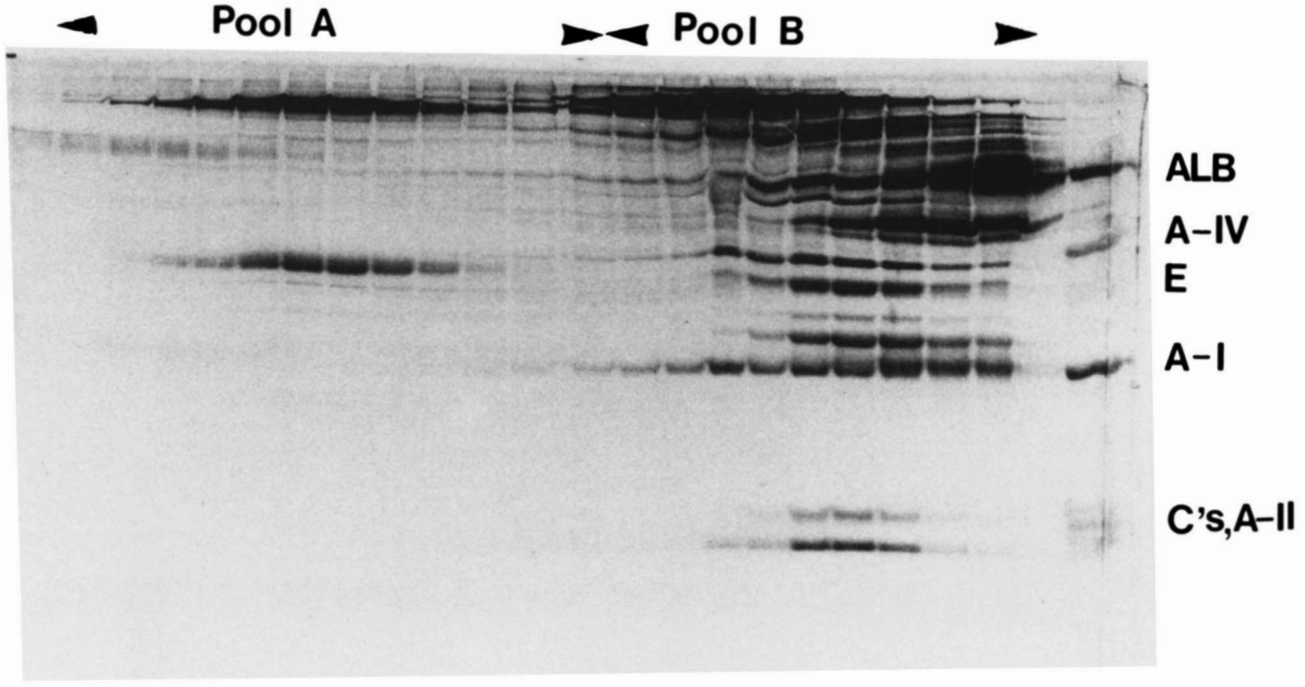

Fig. 3. Localization of C apolipoproteins (and apoA-II) in Bio-Gel A-5m gel-filtered serum. Aliquots (25 μl) of column fractions were analyzed on 12.5% polyacrylamide gels. Pool A eluted from the agarose column between 205 and 235 ml whereas pool B eluted between 240 and 274 ml as indicated in Figs. 1 and 2. Purified bovine serum albumin (BSA), rat apoA-I, apoA-IV, and apoE, as well as a mixture of apoCs and apoA-II were run in the right lane.

TABLE 2. ApoA-I-HDL isolated by agarose column chromatography and immunoprecipitation

Fraction	Original HDL[a] (Pool B)	Fraction Precipitated with Anti-ApoA-IV and Anti-ApoE	Fraction Subsequently Precipitated with Anti-ApoA-I[b]
ApoA-I	103.1 ± 15.8[c]	23.8 ± 7.2	73.3 ± 13.1
ApoA-IV	24.3 ± 1.8	23.7 ± 3.0	0.9 ± 0.8
ApoE	12.2 ± 0.2	11.5 ± 0.9	0.6 ± 1.0
Cholesterol	86.9 ± 5.4	37.1 ± 4.3	56.2 ± 5.3[d]
Phospholipids	127.7 ± 18.3	47.8 ± 7.3	80.5 ± 95.4

[a]Fractions containing the bulk of apoA-I and apoA-IV were pooled (e.g., Fig. 1: 240–274 ml).
[b]ApoA-I, apoA-IV, and apoE were not detectable in the final supernatant.
[c]Results are expressed as μg/ml column fraction. Values are mean ± SD (n = 3).
[d]Of the total cholesterol, 18.4 ± 2.6% was present as unesterified cholesterol.

periments (9) indicated that apoA-IV and apoE in pool B are not present on one and the same HDL particle.

Table 4 summarizes the results of the present study. We calculated the fraction of the total cholesterol present in the various subclasses, assuming that the two pooled column fractions together comprise all lipoproteins present in the HDL size range. In fact it can be calculated that these two pools contain more than 90% of the total cholesterol found in the HDL size range. ApoA-I-HDL and LpE each accounted for as much as 39% of the cholesterol and therefore both are major lipoprotein classes. Sixty-nine percent of the total apoA-I and 63% of the total apoE are present on apoA-I-HDL and LpE, respectively. ApoA-IV-HDL is a minor HDL subclass accounting for only 4% of the total cholesterol. Only a relatively small fraction (about 18%) of the cholesterol found in the HDL size range is present on complex lipoprotein particles. It can be calculated, on the basis of the data shown in Tables 1–3 and earlier experiments (9), that these more complex lipoproteins will consist mostly of particles containing both apoA-I and apoA-IV and particles containing both apoE and apoA-I.

DISCUSSION

In the present study, data are presented in favor of the existence of five different lipoprotein subclasses found in the HDL size range in rat serum. The evidence was obtained by using a combination of gel filtration and specific immunoprecipitation. In a previous study (9), preliminary evidence was obtained suggesting the existence of HDL subfractions, containing either only apoA-I or only apoA-IV. The present experiments were designed to supplement and extend these findings.

An important observation in the present report is that the separation on 6% agarose columns of rat serum lipoproteins in the LDL/HDL size range is improved, compared to our earlier data (9). Two major subfractions in the HDL size range can be easily distinguished now, just by measuring cholesterol in the eluted fractions. Two minor cholesterol peaks are attributed to VLDL and LDL. This is due to the use of different column size and a lower flow rate. The four peaks in the cholesterol profiles were obtained using five different sera and two different columns, indicating that the column chromatography technique is reproducible.

Apolipoproteins were determined by electroimmunoassay, both in the precipitates and in the final supernatants resulting after immunoprecipitation. The precipitates already contained antibody and therefore the theoretical possibility existed for underestimation of the apolipoprotein concentrations in the immunoprecipitates. The recoveries of apoA-IV, apoE, and apoA-I were, however, 99 ± 9%, 100 ± 9%, and 99 ± 10%, respectively (Tables 1–3). This indicates that the antibodies present in the precipitates did not interfere with the apolipoprotein assays. It can be ex-

TABLE 3. ApoA-IV-HDL was isolated by agarose column chromatography and immunoprecipitation

Fraction	Original HDL[a] (Pool B)	Fraction Precipitated with Anti-ApoE and Anti-ApoA-I	Fraction Subsequently Precipitated with Anti-ApoA-IV[b]
ApoA-I	103.1 ± 15.8[c]	97.6 ± 13.5	0.9 ± 0.2
ApoA-IV	24.3 ± 1.8	19.0 ± 2.7	5.1 ± 1.8
ApoE	12.2 ± 0.2	10.7 ± 0.6	0.6 ± 0.6
Cholesterol	86.9 ± 5.4	81.0 ± 6.2	2.4 ± 1.1[d]
Phospholipids	127.7 ± 18.3	125.3 ± 16.0	3.2 ± 3.6

[a]Fractions containing the bulk of apoA-I and apoA-IV were pooled (e.g., Fig. 1: 240–274 ml).
[b]ApoA-I, apoA-IV, and apoE were not detectable in the final supernatant.
[c]Results are expressed as μg/ml column fraction. Values are mean ± SD (n = 3).
[d]Of the total cholesterol, 57 ± 16% was present as unesterified cholesterol.

pected that the high concentrations of urea (8 M) used for the sample preparation caused a dissociation of the apolipoprotein-IgG complexes.

The existence of HDL subclasses in rat serum has been known for some time. Weisgraber, Mahley, and Assmann (6) isolated an apoE-rich HDL (HDL_1) from serum of normal rats and another apoE-rich HDL (HDL_c) from serum of cholesterol-fed rats. These apoE-rich lipoproteins also contained small amounts of apoA-I. Heparin-Sepharose column chromatography (7) had also been used successfully for the isolation of apoE-poor and apoE-rich HDL fractions from rat serum. It should be noted, however, that all these procedures require prior ultracentrifugal isolation of HDL (6, 7). Because ultracentrifugation results in "stripping" of apoA-I and especially apoE from serum HDL (8, 9, 18–21), it is important to avoid ultracentrifugation during the isolation of specific HDL subfractions. The data presented in this paper show that a combination of gel filtration and immunoprecipitation may be very useful.

A major conclusion from the present experiments is that, even in serum from rats fed normal chow (which is free of cholesterol), more than 60% of the total serum apoE is present on a lipoprotein particle in the HDL size range that has apoE as its sole apolipoprotein constituent (LpE). It should be noted that more than 80% of rat serum total cholesterol is present in the HDL size range. Keeping this in mind, it is evident that the quantitative importance of LpE is endorsed by the fact that as much as 39% of the cholesterol is present in this subclass. Lipoprotein particles containing apoE as their sole apolipoprotein (so-called apoE-HDL_c) have also been isolated by Mahley, Weisgraber, and Innerarity (22) from serum obtained from cholesterol-fed dogs. However, a comparison of the composition of both particles reveals several differences. Canine apoE-HDL_c (isolated from the density range 1.006–1.02 g/ml) is a particle with a molecular weight of 3.6×10^6, which is even larger than both human and canine LDL (23). On 6% agarose columns, therefore, these particles elute before the LDL peak (see Fig. 2B). Rat LpE, on the other hand, clearly elutes after the LDL fraction and is therefore smaller in size (mol wt approximately 750,000). It is calculated that canine apoE-HDL_c contains sixteen apoE molecules per particle (24). In contrast, it can be calculated from our data, assuming a molecular weight of 34,000 for apoE and taking into account the fact that 12% (by weight) of rat LpE is apoE protein as measured by electroimmunoassay (Table 1), that only three apoE molecules are present on an average LpE particle. No low molecular weight apolipoproteins can be detected in the column fractions from which LpE was isolated (see Fig. 3). The data given in Table 1 allow us to calculate that LpE has a chemical composition [12% protein, 41% cholesterol(ester), 47% phospholipid (by weight)] that is compatible with a lipoprotein of density < 1.019 g/ml. It is not clear whether ultracentrifugation allows the isolation of LpE in the VLDL or LDL density range. However, it is likely that ultracentrifugal isolation causes redistribution of apoE as well as apoA-I.

Very low levels of LDL are present in rat serum. Consequently the major cholesterol-carrying lipoproteins are found in the HDL size range in this species. We suggest that LpE, present in normal rat serum, plays an important role in the transport of plasma cholesterol to the liver. Several arguments in favor of this hypothesis can be given. Firstly, in vivo studies using HDL specifically labeled in apoE (25, 26) have also shown that the liver is the major degradation site for HDL apoE, while the kidneys are most active in HDL apoA-I degradation (25–27). These in vivo turnover studies support the notion that apoA-I and apoE are not always together on the same HDL particle and that the metabolic pathways of HDL apoE and HDL apoA-I are not identical. Secondly, we observed that LpE does not contain C peptides. Studies of Van Berkel et al. (28) and Windler, Chao, and Havel (29) have shown that C peptides inhibit the hepatic uptake of apoE-containing lipoproteins. The absence of C peptides on LpE is therefore in favor of a role for this simple lipoprotein in reverse cholesterol transport. Thirdly, HDL_c, isolated from plama of cholesterol-fed dogs which also has apoE as its sole apolipoprotein component, binds with a very high affinity to hepatic lipoprotein receptors (30, 31). The high affinity interaction between HDL_c and the apoB,E receptor was shown to be caused by multiple interactions between four sites on the receptor protein and four apoE molecules

TABLE 4. Distribution of total cholesterol, apoA-I, apoE, and apoA-IV among the different lipoprotein subclasses present in the total HDL size range, identified by specific immunoprecipitation

Fraction	Cholesterol	ApoA-I	ApoE	ApoA-IV
		%		
Simple lipoproteins				
LpE	39 ± 4[a]		63 ± 3	
ApoA-I-HDL	39 ± 8	69 ± 8		
ApoA-IV-HDL	4 ± 1			21 ± 8
Complex lipoproteins				
HDL with apoA-I and apoE	18 ± 10	31 ± 8	37 ± 3	
HDL with apoA-I and apoA-IV				79 ± 8

[a]Values are means ± SD (n = 3).

present on the HDL_c particle (22). A second hepatic receptor probably exists for the binding and uptake of chylomicron remnants. This receptor also interacts with canine HDL_c (32). These observations strongly suggest that HDL_c is capable of delivering serum cholesterol to the liver; from there it can be excreted in the bile or degraded to bile acids. A similar mechanism can be expected to operate for LpE present in serum of normal rats as described in the present study.

The second major HDL subclass described in the present paper does not have any apoE or apoA-IV, but contains mostly apoA-I (apoA-I-HDL). Sixty-nine percent of the total HDL apoA-I and 39% of the total HDL cholesterol are present in this subclass. ApoA-I-HDL has a particle weight of about 250,000; the chemical composition resembles that of typical rat HDL. It can be calculated from the data shown in Table 2, assuming that apoA-I is the only apolipoprotein component, that apoA-I-HDL consists of 34% protein, 26% cholesterol(esters), and 40% phospholipids (weight %). Consequently, approximately three apoA-I molecules can be accommodated on one apoA-I-HDL particle.

ApoA-IV is the third major apolipoprotein present in rat HDL (9, 14, 33). In a previous publication (9), we suggested that a small part of the apoA-IV present in the HDL size range was probably located on a protein-rich subclass, containing neither apoE nor apoA-I. The data of the present study provide direct evidence in favor of the existence of this subclass that was called apoA-IV-HDL. If apoA-IV is the only apolipoprotein present on apoA-IV-HDL, the chemical composition follows from the data given in Table 3: 48% protein, 22% cholesterol(esters), and 30% phospholipid (weight %). However, the major part of the total HDL apoA-IV (79%) is present on an HDL particle containing apoA-I as well as apoA-IV, but no apoE. As indicated above for the simple lipoproteins, it can be calculated (assuming a particle weight of 250,000) that two molecules of apoA-IV and one molecule of apoA-I are likely to be accommodated on this complex lipoprotein. Metabolic studies with HDL, specifically labeled in apoA-IV or apoA-I, have shown that the serum half-life of HDL apoA-IV is much shorter than that of HDL apoA-I; the liver is the major catabolic site of HDL apoA-IV and only a minor fraction of total HDL apoA-IV is degraded in the kidneys (36). Our hypothesis is that apoA-IV-HDL will have a relatively rapid turnover, compared to the apoA-IV present on the more complex HDL subclass, containing both apoA-IV and apoA-I, due to a specific degradation of apoA-IV-HDL in the liver. This would indicate a role of apoA-IV-HDL in reverse cholesterol transport. Earlier studies of DeLamatre et al. (17) have already implicated a role of apoA-IV in reverse cholesterol transport, based on the observation that active lecithin:cholesterol acyltransferase causes a redistribution of apoA-IV between "free" apoA-IV and HDL apoA-IV. Our observation that the cholesterol present on apoA-IV-HDL is mainly (57%) unesterified cholesterol supports the idea of a specific role of this HDL subfraction in cholesterol transport. The relatively low fraction of esterified cholesterol is consistent with a location of apoA-IV-HDL at the start of overall reverse cholesterol transport as well as with rapid turnover of this HDL subfraction.

Finally, our data suggest the existence of a second complex HDL subclass, containing both apoE and apoA-I. Thirty-seven percent of the apoE present in total rat HDL isolated from chow-fed rats was found to be present in combination with apoA-I. At the moment it is impossible to indicate the exact chemical composition of this complex HDL subclass. Assuming a particle weight of about 300,000, three apoA-I molecules and one apoE molecule could be accommodated on a particle with 40% protein.

In conclusion, the data presented in the present paper draw a new picture of the rat lipoproteins found in the HDL size range. Five subclasses can be distinguished, including three relatively simple lipid protein complexes containing only one major HDL apolipoprotein each (apoA-I-HDL, apoA-IV-HDL and LpE), and two complex lipoproteins, containing either both apoA-I and apoA-IV or apoE as well as apoA-I. ApoCs or apoA-II are not present on LpE, but may be present on one or more of the other lipoprotein subclasses.

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