Distribution of apolipoprotein A-IV among lipoprotein subclasses in rat serum

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Abstract The distribution of apolipoproteins (apo) A-I, A-IV, and E in sera of fed and fasted rats was studied using various methods for the isolation of lipoproteins. Serum concentrations of apoA-I and apoA-IV decreased significantly during fasting (16 and 31%, respectively), while apoE concentrations remained essentially the same. Chromatography of sera on 6% agarose columns showed that apoA-IV is present on HDL and as so-called "free" apoA-IV. The concentration of "free" apoA-IV decreased six- to seven-fold during fasting, explaining the decrease in total serum apoA-IV. Serum apoA-I and apoE are almost exclusively associated with HDL-sized particles. When sera are centrifuged at a density of 1.21 g/ml, marked quantities of apoA-I (8–9%) and apoE (11–22%) are recovered in the "lipoprotein-deficient" infranatant, suggesting that ultracentrifugation affects the integrity of serum HDL. The nature of the chromatographically separated carriers of serum apoA-IV was investigated by quantitative immunoprecipitation. From these studies, it is concluded that apoA-IV in rat serum is present in at least three fractions: *1*) particles with the size and composition of HDL, containing both apoA-I and apoA-IV and possibly minor quantities of apoE; *2*) HDL-sized particles containing apoA-IV, but no apoA-I or apoE; *3*) "free" apoA-IV, probably containing small amounts of bound cholesterol and phospholipid. — **Dallinga-Thie, G. M., P. H. E. Groot, and A. van Tol.** Distribution of apolipoprotein A-IV among lipoprotein subclasses in rat serum. *J. Lipid Res.* 1985. **26:** 970–976.

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Apolipoprotein A-IV (apoA-IV) was first described by Swaney, Braithwaite, and Eder (1) as a polypeptide with a molecular weight of 46,000 present in rat HDL. The distribution of apoA-IV in human and rat serum is distinct from that of apoA-I and apoE, as apoA-IV is present in HDL as well as in a so-called "free" form (2–5). The possible metabolic relationship between apoA-IV present in HDL and non-lipoprotein-bound apoA-IV has been investigated using ultracentrifugation to separate both pools of apoA-IV (6). Because it is known that HDL apoA-I and apoE will dissociate from HDL during ultracentrifugation (7–10), we compared the effect of the isolation procedure (gel filtration versus ultracentrifugation) on the distribution of apoA-IV, apoA-I, and apoE. We also isolated the various fractions carrying apoA-IV in serum by agarose gel chromatography, combined with immunoprecipitation. This report describes the existence of three fractions in serum containing apoA-IV, two HDL species and a "free" apoA-IV probably containing small amounts of bound cholesterol and phospholipid.

MATERIALS AND METHODS

Serum samples

Male Wistar rats, weighing 250–300 g, maintained on standard laboratory chow and tapwater were used. Blood was collected at 9 AM from fed or 20-hr food-deprived animals by aortic puncture under light ether anesthesia. The blood was kept on ice for 2 hr. The 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.

Gel filtration of serum

Ten ml of pooled serum from fasted or fed rats, containing 10% sucrose (w/v) was applied on a 6% agarose column (2.5 cm × 120 cm, Bio-Rad, Richmond, CA), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate buffer (pH 7.4), 0.01% NaN_3, and 1 mM EDTA (11), operated at 4°C. The flow rate was 15–20 ml/hr and fractions of 5 ml were collected. The column was calibrated with iodinated rat serum albumin, rat serum VLDL and HDL, and with human LDL. Recoveries of cholesterol, apoA-I, apoA-IV, and apoE were

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; EDTA, ethylenediamine tetraacetic acid; IgG, γ-immunoglobulins.

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96 ± 4%, 87 ± 12%, 91 ± 7%, and 80 ± 2%, respectively (means ± SD, n = 4).

Ultracentrifugal isolation of serum lipoproteins

Serum of fasted or fed rats was adjusted to density 1.21 g/ml with solid KBr. Aliquots (3 ml) of these sera were carefully overlayered with KBr solution of density 1.21 g/ml containing 1 mM EDTA. The lipoprotein-containing ($d < 1.21$ g/ml) and the lipoprotein-deficient ($d > 1.21$ g/ml) fractions were isolated in the Beckman 40.3 rotor operating at 12°C and 38,000 rpm for 48 hr. The recoveries of cholesterol, apoA-I, apoA-IV, and apoE were 98 ± 6%, 97 ± 13%, 102 ± 9%, and 91 ± 13%, respectively (means ± SD, n = 8).

Immunoassays

ApoA-I, apoA-IV, and apoE concentrations were determined by the electroimmunoassay technique of Laurell (12), as modified by Dallinga-Thie, Groot, and van Tol (13).

Quantitative immunoprecipitation

Rabbit anti-apoA-I and anti-apoA-IV IgG, prepared as described (13), were used for the quantitative immunoprecipitation of apoA-I- and apoA-IV-containing lipoproteins. Immunoprecipitations were performed by adding predetermined quantities of the IgG's to the apoA-IV-containing fractions, obtained by 6% agarose column chromatography of sera. Mixtures were incubated overnight at 4°C and the immunoprecipitates were isolated by low speed centrifugation at 4°C. Apolipoprotein concentrations were determined in both the precipitate and supernatant. Both supernatant and precipitate were extracted according to Bligh and Dyer (14) in order to determine the lipid composition. The chloroform phase was evaporated under a stream of nitrogen and used for lipid determinations. Cholesteryl esters were saponified with alcoholic KOH. After extraction with petroleum ether, total cholesterol was determined as described. Appropriate cholesterol standards were run in parallel throughout the whole procedure. The amount of cholesterol precipitated by a control rabbit IgG preparation isolated from a non-immunized rabbit was negligible.

Chemical analysis and statistics

Total cholesterol was determined with an enzymatic method (CHOD-PAP kit, Boehringer, Mannhein, F.R.G.; cat. no. 310328). Total phospholipid phosphorus was determined according to Bartlett (15). Prior to this procedure, samples were precipitated, in the presence of albumin as a carrier, with trichloroacetic acid (final concentration 5%, w/v). The precipitate was collected by centrifugation.

The data collected in this study were statistically evaluated using Student's unpaired, two-tailed *t* test.

RESULTS

Effects of nutritional status on serum lipid and apolipoprotein concentrations

In **Table 1** lipid and apolipoprotein concentrations in sera from fed and fasted rats are presented. The serum cholesterol levels were not affected by the nutritional status, but total serum apoA-IV concentration decreased significantly during a 20-hr fasting period. After more prolonged fasting (48 hr) the serum apoA-IV concentration was further reduced to 5 mg% (unpublished observation). Fasting also resulted in a minor, but statistically significant, decrease in serum apoA-I concentration, but the apoE level remained essentially the same.

Gel filtration of sera from fed and fasted rats

Figs. 1 to 4 show the elution patterns of apolipoproteins and cholesterol in rat serum fractionated on 6% agarose gel columns. A minor cholesterol peak is eluted in the void volume together with the triglycerides (not shown), consisting of chylomicrons and/or VLDL. The major peak in cholesterol is largely due to HDL because the LDL level in rat serum is low. There is no difference in total serum cholesterol concentration between sera from fed and fasted rats, but a skewed major peak in cholesterol is present in the fed state, indicating more variety in particle size (compare **Fig. 1A** and **Fig. 2A**). This phenomenon is not due to an increase in LDL in the fed state as LDL is eluted at a smaller elution volume (indicated by the horizontal bars, representing 90% of human LDL applied to the column in separate experiments, in Fig. 1A and 1B) as determined in separate experiments. The peak in absorbance at 280 nm coinciding with the shoulder of the cholesterol peak (fractions 70–80) is due to immunoglobulin M (mol wt 900,000).

The distributions of apoA-I and apoE are shown in Figs. 1B and 2B and those of apo-IV in **Fig. 3** and **Fig. 4.** In the fed state a minor fraction of apoA-I and apoE is located in the VLDL/chylomicron peak, while the majority (>90%) is found in the HDL peak. The elution

TABLE 1. Cholesterol, apoA-I, apoA-IV, and apoE concentrations in sera of fed and fasted rats

	Fed[a]	Fasted[a]
	mg/dl ± SD	
Cholesterol	55.0 ± 6.0	53.0 ± 6.0
ApoA-I	39.8 ± 5.7	33.3 ± 3.1[b]
ApoA-IV	15.7 ± 2.0	10.8 ± 1.7[c]
ApoE	22.0 ± 5.0	24.5 ± 4.6

[a]Six experiments.
[b]Statistically significant at $P < 0.05$.
[c]Statistically significant at $P < 0.001$.

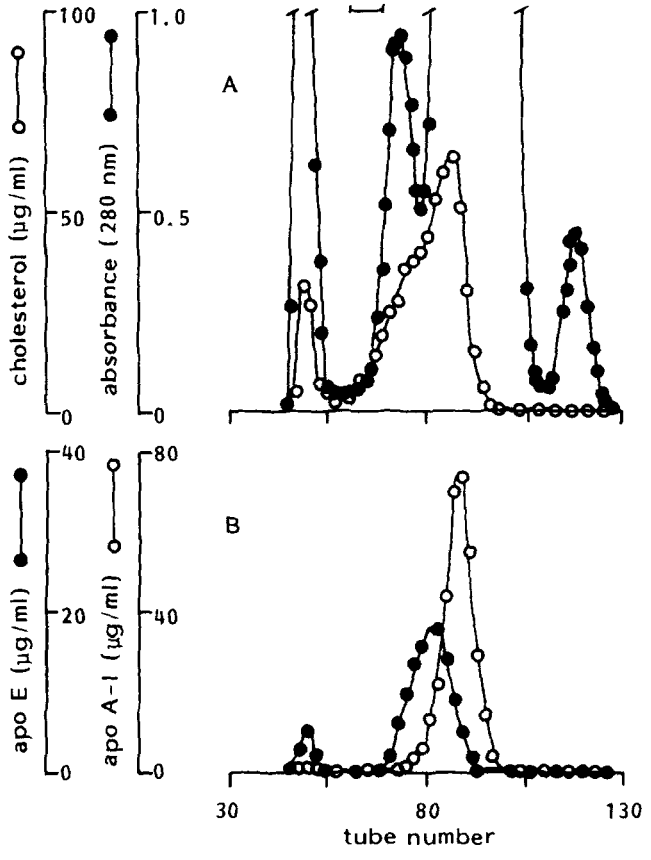

Fig. 1. Separation on a column of 6% agarose gel of serum from chow-fed rats. Ten ml of serum was applied to a column of 6% agarose (2.5 cm × 120 cm), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate, pH 7.4, 1 mM EDTA, and 0.1% NaN_3 at 4°C. The horizontal bar indicates the elution volume of human LDL.

profiles of apoE and apoA-I in the HDL region were not identical as apoE clearly preceded apoA-I. No big differences in elution patterns of apoE or apoA-I were observed between the fed and fasted states. Although comparison of Figs. 1B and 2B suggests an increase in HDL apoE after fasting, this observation appeared to be statistically insignificant. In sera from fasted animals, apoE was present in VLDL while no apoA-I could be detected in this fraction (Fig. 2B). In sera from fed animals, both apoA-I and apoE were detectable in the chylomicron/VLDL peak, although the amount of apoA-I in the void volume was extremely low (Fig. 1B).

With a 6% agarose column it is possible to obtain a clear separation between two distinct fractions containing apoA-IV (Figs. 3 and 4). The first peak of apoA-IV elutes together with apoA-I and these fractions also contain the bulk of the serum cholesterol. The second apoA-IV peak elutes together with the bulk of the serum proteins (and iodinated albumin; not shown) in fractions containing little cholesterol, no apoA-I, and no apoE. In the fed state, an average of 36 ± 4% of the total apoA-IV mass was found in this second peak compared to 9% in the fasted state (**Table 2**). The amount of apoA-IV recovered in the size-range of typical HDL is not influenced, indicating that the decrease in serum apoA-IV level after a 20-hr fasting period (Table 1) is due to a decrease in the second apoA-IV peak.

Ultracentrifugation

Data on the cholesterol and apolipoprotein distribution between the d < 1.21 g/ml and d > 1.21 g/ml fractions are presented in **Table 3.** Essentially all cholesterol was recovered in the d < 1.21 g/ml fractions, but significant amounts of serum apoA-IV, apoA-I, and apoE, varying between 8% and 46% for the different apolipoproteins, are found in the "lipoprotein-deficient" (*d* > 1.21 *g/ml*) fraction. The amount of apoA-IV in this lipoprotein-deficient fraction is dependent upon the nutritional status; it decreased from 7.0 mg/dl in fed animals to 3.9 mg/dl after 20 hr of fasting. These values represent 46% and 35% of total serum apoA-IV in the fed and the fasted

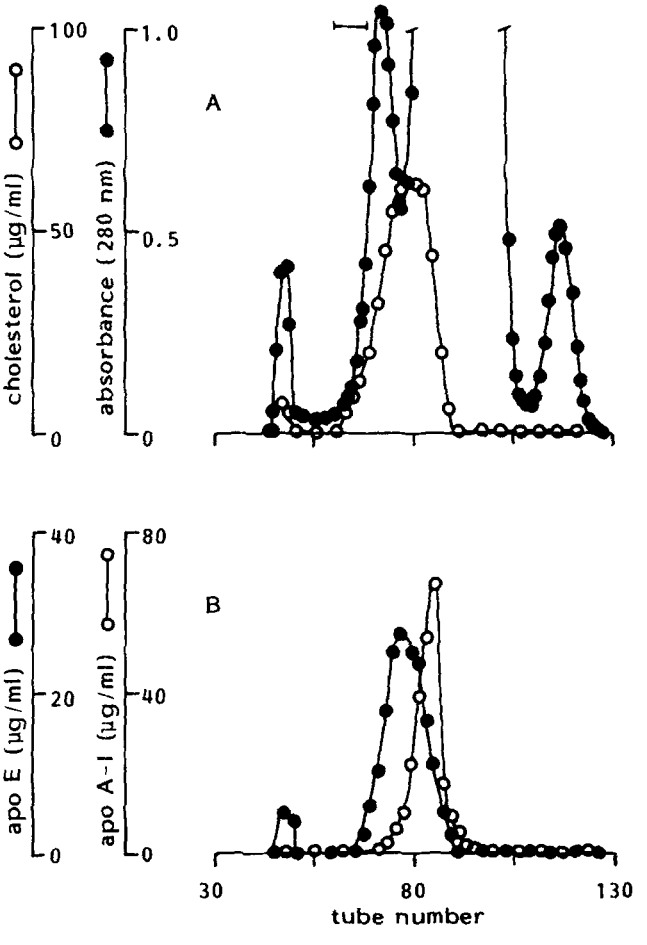

Fig. 2. Separation on a column of 6% agarose gel of serum from rats deprived of food for 20 hr. Ten ml of serum was applied to a column of 6% agarose (2.5 cm × 120 cm), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate, pH 7.4, 1 mM EDTA, and 0.1% NaN_3 at 4°C. The horizontal bar indicates the elution volume of human LDL.

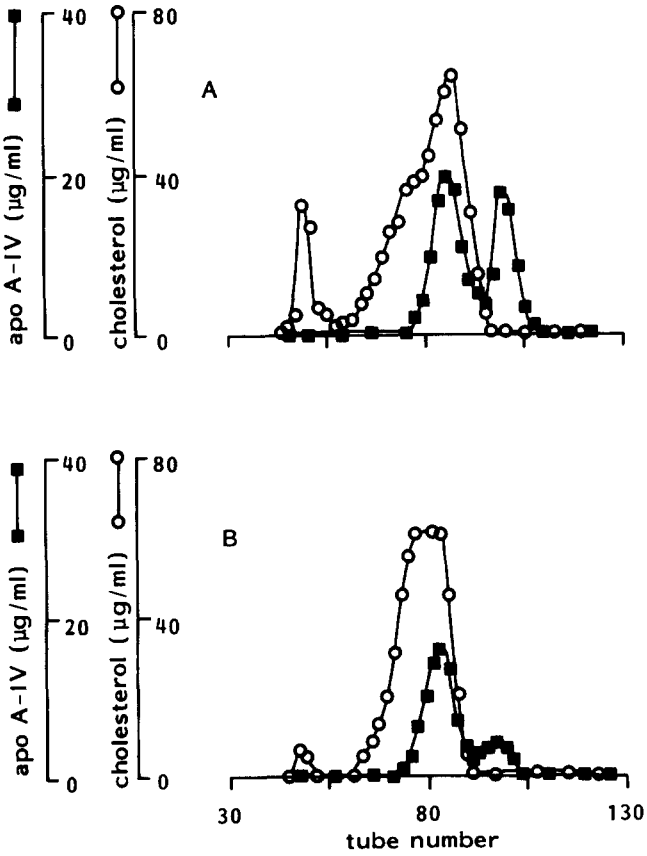

Fig. 3. Distribution of apoA-IV (■) and cholesterol (○) between serum lipoproteins of fed rats (A) and fasted rats (B) separated on a column of 6% agarose gel.

states, respectively. In both cases the amount of apoA-IV found in this lipoprotein-deficient fraction is significantly higher ($P < 0.005$) compared to the amount found in the "free" apoA-IV fraction, using the gel filtration method (see Table 2).

Immunoprecipitation studies

In order to obtain information about the composition of the carriers of apoA-IV in serum, we fractionated serum by agarose gel chromatography and precipitated the apoA-IV-containing particles in the HDL size-range, as well as in the "free" apoA-IV peak, using anti-apoA-IV IgG. Similar studies were performed using anti-apoA-I IgG; immunoprecipitates as well as supernatants were analyzed chemically and immunochemically. The results of these experiments are shown in Tables 4 and 5. It can be concluded from the data in **Table 4** that apoA-IV in serum is associated with at least two species with the size of HDL: *1*) particles containing apoA-I, apoA-IV, and possibly minor quantities of apoE; and *2*) particles containing apoA-IV, but neither apoA-I nor apoE.

These conclusions are based on the partial precipitation of apoA-IV (60%) using anti-apoA-I, indicating that about 40% of apoA-IV in HDL is not associated with apoA-I. Furthermore, insignificant quantities of apoE were precipitated using anti-apoA-IV, in line with the existence of an apoA-IV-containing HDL, free of apoA-I and apoE. The chemical composition of HDL fractions precipitated with anti-apoA-I is similar to that of serum HDL, e.g., a protein content of 35–40% and a phospholipid:cholesterol ratio of 2:1. HDL-sized fractions precipitated with anti-apoA-IV seem to have a higher protein content and a phospholipid:cholesterol ratio closer to 1 (Table 4). Only about 6% of HDL apoA-I can be precipitated with anti-apoA-IV, indicating that more than 90% of HDL apoA-I is present on an HDL subfraction deficient in apoA-IV, assuming a particle weight for HDL of about 275,000. It can also be calculated that the bulk of this HDL is deficient in apoE because the amount of apoE precipitated by anti-apoA-I is very low.

Similar analyses of the "free" apoA-IV fraction (see **Table 5**) revealed that small amounts of cholesterol and phospholipid are associated with apoA-IV (about 11% by weight). Neither apoE and apoA-I (checked by immunoassay) nor apoC and apoA-II (checked by SDS-polyacrylamide gel electrophoresis) could be detected in the immunoprecipitate.

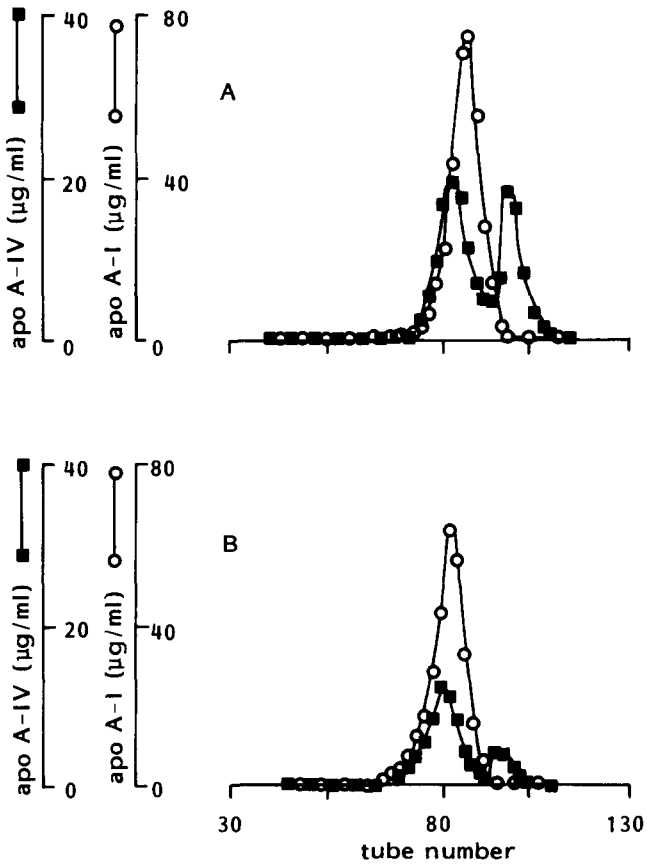

Fig. 4. Distribution of apoA-IV (■) and apoA-I (○) between serum lipoproteins of fed rats (A) and fasted rats (B) separated on a column of 6% agarose gel.

TABLE 2. Distribution of apoA-IV in serum between HDL and "free" apoA-IV, separated by 6% agarose chromatography

	HDL (First ApoA-IV Peak)	"Free" ApoA-IV (Second ApoA-IV Peak)
	mg/dl	
Fed[a]	8.7 ± 1.6	4.9 ± 0.4 (36 ± 4%)[b]
Fasted[c]	8.5; 7.0	0.8; 0.7 (8–10%)[d]

[a]Mean values of four experiments ± SD.
[b]Numbers in parentheses are % recoveries of total serum apoA-IV in the second peak.
[c]Values of two separate experiments.
[d]Significantly lower than the value in serum obtained from fed animals, $P < 0.005$.

DISCUSSION

Rat apoA-IV is mainly synthesized in the intestine (16, 17) and its concentration in serum is affected by the dietary history of the animal. Studies of DeLamatre and Roheim (3) have shown that single meal feeding of rat chow, a high fat meal, or a high fat meal plus cholesterol results in a 40–80% increase in serum apoA-IV, with no observed effect on serum apoA-I. Long-term feeding of a cholesterol/high fat diet also results in an increase in serum apoA-IV compared to a chow-fed control group. The data presented in this report show that the levels of apoA-IV in sera of fasted rats are decreased by 31% compared to postprandial levels in chow-fed rats.

Chylomicrons in intestinal lymph carry both apoA-I and apoA-IV. After entering the plasma compartment, part of the apoA-I rapidly dissociates from the chylomicrons and is transferred to HDL (18–21); the same mechanism has been proposed for apoA-IV (19, 21). It appears from our data that the dissociation of apoA-IV from the chylomicrons is even more complete than for apoA-I, as apoA-IV could not be detected at all in the chylomicron/VLDL fraction after analysis of serum from fed rats on 6% agarose columns (Fig. 3). More recently it has been suggested that the precursor–product relationship between chylomicron- and HDL-apoA-IV is not direct, but linked by a passage though the pool of "lipoprotein-free" apoA-IV (6).

We recovered 9% of serum apoA-I, the main protein of HDL, in the d > 1.21 g/ml fraction after one ultracentrifugal run of 24 hr, which is in agreement with reported values (22). No apoA-I could be detected in column fractions following the HDL peak during fractionation of serum by gel filtration (Table 2, Figs. 1, 2, and 4). It was reported that about 50% of the apoE was lost to the "lipoprotein-free" infranatant during a standard isolation of HDL by sequential ultracentrifugation (8, 22, 23). In the present study only one ultracentrifugal step was performed leading to "stripping" of about 11–22% of serum apoE. All fractions containing apoE or apoA-I on gel filtration of serum also had substantial amounts of cholesterol. Although the existence of lipid-free aggregates of apolipoproteins with the size of HDL and a density > 1.21 g/ml cannot be excluded, this suggests an artificial origin of apoE and apoA-I in the ultracentrifugal "lipoprotein-deficient" fraction. The underlying cause of this proposed ultracentrifugal loss of lipoprotein apolipoproteins is complex and probably attributable to combined effects of high ionic strength and high sheering forces (10).

The situation for apoA-IV is clearly different. Fractionation of rat serum by gel filtration results in the recovery of part of the apoA-IV in a fraction that elutes after the main cholesterol peak. The concentration of this "free" apoA-IV is very dependent upon the nutritional status of the animal. It can be calculated from data in Table 2 that there is a six- to seven-fold decrease in "free" apoA-IV

TABLE 3. Distribution of cholesterol, apoA-I, apoA-IV, and apoE after centrifugation of serum at a density of 1.21 g/ml

	Total Serum	d < 1.21 g/ml		d > 1.21 g/ml	
	mg/dl ± SD (n = 4)[a]				
Fed					
Cholesterol	51.5 ± 3.2	50.5 ± 3.3		n.d.[b]	
ApoA-I	42.2 ± 5.4	35.0 ± 4.0	(83 ± 9%)	3.4 ± 0.4	(8 ± 1%)
ApoA-IV	15.3 ± 1.9	9.1 ± 0.5	(59 ± 3%)	7.0 ± 0.6	(46 ± 4%)
ApoE	20.0 ± 4.4	13.4 ± 4.0	(66 ± 12%)	4.4 ± 0.8	(22 ± 4%)
Fasted					
Cholesterol	51.3 ± 6.5	50.1 ± 6.3		n.d.[b]	
ApoA-I	34.4 ± 3.1[c]	31.7 ± 3.0	(91 ± 9%)	3.3 ± 0.9	(9 ± 3%)
ApoA-IV	11.2 ± 0.8[d]	7.0 ± 0.6	(63 ± 5%)	3.9 ± 0.8[d]	(35 ± 7%)
ApoE	22.7 ± 2.3	19.2 ± 2.2	(85 ± 13%)	2.3 ± 1.0	(11 ± 5%)

[a]Numbers in parentheses are the percentage recoveries in the separate fractions.
[b]Not detectable, without prior concentration.
[c]Significantly lower than the values in serum obtained from fed animals, $P < 0.05$.
[d]Significantly lower than values in serum obtained from fed animals, $P < 0.005$.

TABLE 4. Analysis of HDL isolated by chromatography of rat serum on 6% agarose columns

	HDL[a]	HDL Precipitated with Anti-ApoA-I		HDL Precipitated with Anti-ApoA-IV	
Cholesterol	79.8 ± 1.2[b]	58.9 ± 0.2		12.0 ± 2.1	
Phospholipids	142.8 ± 5.7	113.3 ± 2.5		15.4 ± 1.6	
ApoA-I	79.2 ± 1.5	79.2 ± 1.5	(77.5 ± 6.3)[c]	4.3 ± 2.7	
ApoA-IV	22.3 ± 5.2	14.5 ± 0.9		22.3 ± 5.2	(25.3 ± 5.7)[c]
ApoE	14.6 ± 1.5	4.7 ± 0.8		0.9 ± 1.0	

[a]Fractions containing the bulk of apoA-I (and apoA-IV) were pooled.
[b]Results are expressed as μg/ml column fraction. Values are means ± SD (n = 3).
[c]The values in parentheses were assayed in the precipitates and agreed very well with the amounts present in the original HDL preparation. HDL apoA-I and HDL apoA-IV were quantitatively precipitated using anti-apoA-I or anti-apoA-IV, respectively.

during fasting. Others have shown that cholesterol feeding (3) and aging (4) will result in an increasing amount of apoA-IV in this fraction. In human serum, using either ultracentrifugation or crossed immunoelectrophoresis, it was observed that even more (>95%) of the serum apoA-IV was present in a form not bound to VLDL, LDL, or HDL (2, 24). Using gel filtration chromatography, about 77% of human apoA-IV was found in fractions recovered from the column after VLDL, LDL, and HDL (5). In our experiments, 46% and 35% (in serum from fed and fasted rats, respectively) of the total serum apoA-IV were recovered in the d > 1.21 g/ml fraction after one ultracentrifugal run at d 1.21 g/ml. Using agarose column chromatography, 36% and 9% (fed versus fasted; see Table 2) of serum apoA-IV were found in the "free" apoA-IV fraction, indicating that not only apoA-I or apoE but also apoA-IV may be "stripped" from rat HDL during ultracentrifugation.

Results of our immunoprecipitation experiments showed that the "free" apoA-IV fraction contained small amounts of bound cholesterol and phospholipid, which were detectable after precipitation with anti-apoA-IV. In these experiments the "free" apoA-IV fraction was first treated with anti-apoA-I, in order to exclude any contamination of HDL-sized particles containing apoA-I. However, some contamination of "free" apoA-IV with apoA-IV-containing lipoproteins with the size of HDL cannot be excluded (see below).

Further characterization of apoA-IV-containing particles with the size of HDL was performed by precipitation with specific antisera against apoA-I and apoA-IV. From our experiments it can be concluded that, based on apolipoprotein composition, HDL is heterogeneous. It is concluded that two apoA-IV-containing HDL particles are present in rat serum: *1*) particles that have apoA-IV, but no apoA-I and apoE; and *2*) particles that contain both apoA-I and apoA-IV and possibly some apoE. In addition, the data suggest that the major part of HDL apoA-I is present on a particle that contains neither apoA-IV nor apoE.

More research is needed for further characterization of the different HDL subfractions and for definition of their origin and specific metabolic functions.

TABLE 5. Chemical composition of the "free" apoA-IV fraction isolated by chromatography of rat serum on 6% agarose columns, followed by immunoprecipitation

Cholesterol	0.9 ± 0.7[a]
Phospholipid	0.6 ± 0.4
ApoA-I	0
ApoA-IV	10.2 ± 2.8 (12.2 ± 3.4)[b]
ApoE	0

[a]The fractions containing the bulk of the "free" apoA-IV, obtained from serum of fed rats by agarose gel filtration, were first treated with anti-apoA-I in order to remove any contaminating apoA-I-containing lipoproteins. The resulting "free" apoA-IV fraction was subsequently precipitated with anti-apoA-IV. Lipids, apoA-I, and apoE were measured in the precipitate. Results are expressed as μg/ml column fraction. Values are means ± SD (n = 3).
[b]ApoA-IV was measured both in the "free" apoA-IV fractions directly obtained from the agarose columns and in the final precipitate (values in parentheses).

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