Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat

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Abstract The feeding of cholesterol to rats resulted in marked alterations in the type and distribution of the plasma lipoproteins and their apoproteins. The hyperlipoproteinemia was characterized by an increase in the d < 1.006 lipoproteins (B-VLDL and VLDL), an increase in the intermediate and low density lipoproteins (LDL), and the appearance of HDL_c. Associated with these lipoproteins was a prominence of the arginine-rich apoprotein. The high density lipoproteins (HDL) were decreased. A two-dimensional immunoelectrophoretic procedure was adapted to quantitate the changes in distribution of the arginine-rich apoprotein in the plasma and various ultracentrifugal fractions obtained from control and cholesterol-fed rats. In rats fed the cholesterol diet, the total plasma arginine-rich apoprotein increased from a control value of approximately 29 mg/dl to 47 mg/dl. The method of ultracentrifugation, however, was found to markedly alter the quantitative results. When the 60 Ti rotor was used at maximum speed to isolate the ultracentrifugal fractions, less than 50% of the total plasma arginine-rich apoprotein was associated with the lipoproteins in the d < 1.006 or the d 1.006–1.02, 1.02–1.063, or 1.063–1.21 ultracentrifugal fractions. By contrast, after limited ultracentrifugation with the 40 rotor, much less arginine-rich apoprotein was lost, with approximately 20% of the arginine-rich apoprotein in control rats and 10% in cholesterol-fed rats found in the d > 1.21 fraction. Significant alterations in the arginine-rich apoprotein quantitation notwithstanding, the observations of increased arginine-rich apoprotein in the B-VLDL, intermediate fraction, and HDL_c following cholesterol feeding remained valid. However, precise quantitation awaits refinements in lipoprotein isolation techniques.

Supplementary key words Two-dimensional quantitative immunoelectrophoresis · arginine-rich apoprotein · atherogenic hypercholesterolemia

The rat has been shown to be particularly resistant to the development of hypercholesterolemia and atherosclerosis. Hypercholesterolemia sufficient to induce experimental atherosclerosis requires the feeding of diets that contain saturated fat, cholesterol, bile acids, and the antithyroid drug, propylthiouracil (1–3). The purpose of the first part of this study was to characterize the hyperlipoproteinemia of the rat on such a dietary regimen and to compare these results with those obtained in similar studies with other animal models. Previously, we reported marked changes in the plasma lipoproteins associated with the development of atherosclerosis in hypothyroid dogs fed a diet similar to that used here. Resulting changes in the canine lipoproteins included the occurrence of a β-migrating lipoprotein (B-VLDL) in the d < 1.006 fraction, an increase in LDL, and the appearance of a cholesterol-rich lipoprotein referred to as HDL_c which had α mobility and was similar in chemical composition and size to LDL but lacked the B apoprotein (4). We have also shown (5) that euthyroid miniature swine fed a diet containing 15% lard and 1% cholesterol (without bile acids or propylthiouracil) developed similar changes in their lipoproteins, including increased LDL and the occurrence of B-VLDL and HDL_c. Associated with the lipoprotein changes were alterations in the type and distribution of the apoproteins. The most distinctive apoprotein change was a prominence of the arginine-rich apoprotein (ARP) associated with the cholesterol-induced B-VLDL, LDL, and HDL_c. The arginine-rich apoprotein characterized in swine lipoproteins (5) was similar to the protein reported in the cholesterol-fed rabbit (6) and in the human Type III B-VLDL (7).

In the second part of this study an immunochemical method was developed to quantitate the changes in the content and distribution of the argi-

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; HDL_c, cholesterol-induced lipoprotein; B-VLDL, β-migrating very low density lipoproteins.

nine-rich apoprotein of lipoproteins from control and hypercholesterolemic rats. The method of two-dimensional immunoelectrophoresis, in association with the use of detergents for delipidation, has already been shown to be useful in quantitation of various membrane proteins (8, 9). The implementation of this method for use in quantitative analysis of apoproteins was of interest, particularly since changes in protein composition appear to be directly linked to the development of hypercholesterolemia which may precede or accompany atherosclerosis. Validation of this method with respect to the quantitation of the arginine-rich apoprotein is presented.

MATERIAL AND METHODS

Osborne Mendel male rats weighing 200–300 g were maintained under constant temperature–humidity conditions with food and water available at all times. Control rats were fed a commercial pelleted laboratory chow (Ralston Purina). Animals on the cholesterol-rich diet were given the same chow in powdered form to allow mixing with the supplements. The cholesterol-rich diet was prepared by adding lard (Esskay, Baltimore, MD) and cholesterol, taurocholic acid, and propylthiouracil (USP; ICN, Cleveland, OH) to the laboratory chow. This mixture was prepared by liquefying the lard at 60°C, then adding the cholesterol and taurocholic acid slowly and with constant stirring. The hot suspension was thoroughly mixed with the dry chow. The final chow contained 5% lard, 1% cholesterol, 0.1% propylthiouracil, and 0.3% taurocholic acid by weight. Rats were on the diets from 2 to 4 weeks prior to lipoprotein analysis. In studies to determine the total plasma arginine-rich apoprotein content, additional diets containing one or two of the supplements were prepared. The final concentrations of the supplements in these diets were identical with those in the cholesterol-rich diet described above.

Lipoprotein preparation and characterization

Rat plasma was obtained by exsanguination from the terminal aorta after ether anesthesia. All animals were fasted 6–8 hr before sampling between 2:00 and 4:00 PM. Plasma lipoproteins were isolated by ultracentrifugation (10) at various densities between 1.006 and 1.21 using a Beckman 60 Ti rotor (Spinco Div., Palo Alto, CA) at 59,000 rpm in an L2 65B ultracentrifuge. The d < 1.006 and d 1.006–1.02 fractions were isolated by ultracentrifugation for 15 hr and washed by an additional centrifugation at the appropriate density. The d 1.02–1.063 fractions were centrifuged 16–18 hr. The d 1.063–1.21 fractions were centrifuged for 48 hr and washed by recentrifugation for an additional 24 hr. As previously reported (11), where a mixture of lipoproteins occurred in a single density fraction, further purification was achieved by use of Geon–Pevikon block electrophoresis. Purity of the lipoproteins obtained from the block electrophoresis was determined by paper or agarose electrophoresis, immunoelectrophoresis, and negative staining electron microscopy (11, 12). Recovery of total cholesterol in the density fractions, as compared with the plasma values, was 70–75% in the control and cholesterol-fed animals, presumably because of losses during the ultracentrifugal washing of the fractions. These washes were required to eliminate albumin contamination. These losses, which were consistent among the different pools, were greater than previously experienced using the same procedures for the isolation of dog, swine, and monkey lipoproteins and may be a property of rat lipoproteins.

Chemical analysis of the purified lipoproteins and the ultracentrifugal density fractions included total cholesterol (13), triglyceride (14), esterified cholesterol (15), phospholipid (16), and protein (17). Apoproteins were obtained by delipidation as previously described (12) and analyzed by Tris–urea (18) and sodium dodecyl sulfate (19) polyacrylamide gel electrophoresis as reported earlier (5).

Two-dimensional immunoelectrophoresis

Methodology. The technique was a modification of the Davies (20) method, based on previous studies of Minchin Clarke and Freeman (21) and on the original work of Laurell (22). The modular tank system, lint wicks, slide holders, and power supply as described by Davies (20) were purchased from Medical and Biological Instrumentation Ltd., Ashford, Kent, England. Slides were standard 2 × 2 in photographic cover slides. Sea-Kem agarose was obtained from Bausch and Lomb, Rochester, NY; dextran (average mol wt 73,000) from Sigma Chemical Co., St. Louis, MO; and Triton X-100 from Packard Instruments, Palo Alto, CA.

For the first-dimension gel, agarose was dissolved in barbital buffer (ionic strength 0.0375 μ, pH 8.6) to make a 1% solution, which was kept in a water bath at 50°C. To enhance the sensitivity of the method, 2% dextran was added to the agarose solution (23); 0.05% of the nonionic detergent Triton X-100 was also added (8). The warm agarose solution (5.4 ml) was poured onto warm slides, and the gels were allowed to stand in a humidity chamber at room temperature for 45 min.

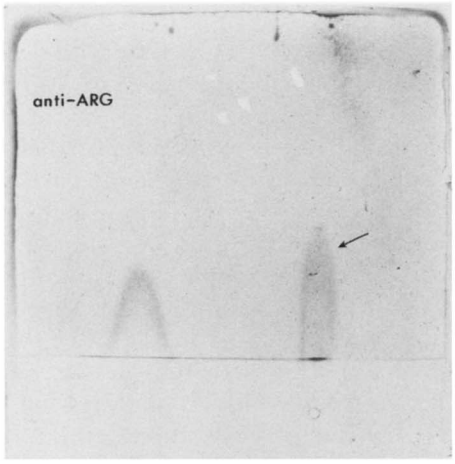

Fig. 1. Immunoprecipitin "rocket" obtained for the arginine-rich apoprotein in normal rat VLDL. The rocket indicated by the arrow was obtained by running in the second dimension only.

For the immunoelectrophoresis studies, ultracentrifugal fractions d < 1.006 and d 1.006–1.02, 1.02–1.063, and 1.063–1.21, not further purified by Geon–Pevikon electrophoresis, were used. The samples, previously dialyzed against distilled water–0.01% EDTA, were incubated for 2 hr at room temperature in 1% Triton X-100. A 1-mm sample well was punched 6.5 mm from one corner of each gel with a stainless steel tubular cutter, and the plug was removed. With a sharp razor, a slab of gel was removed from each slide, leaving a 1.2 × 5 cm strip (first-dimension gel) with the sample well in one corner. A 1-μl lipoprotein sample, stained with bromphenol blue marker, was applied with a modified Hamilton 1-μl syringe.

The slides were mounted gel side down on wicks laid on the plastic holders. The wicks were presoaked in buffer and squeezed of excess liquid. They extended 1–2 cm into the buffer chambers and ~0.5 cm onto the gel surface. Each buffer chamber contained 240 ml of barbital buffer (Buffer B-2, Beckman Instruments; diluted 1 package/2 liters, ionic strength 0.0375 μ, pH 8.6) containing 2% dextran and 0.05% Triton X-100. A voltage gradient of 5 V/cm was applied for 2 hr.

The antiserum gel (second-dimension gel) was prepared with dextran and Triton X-100 as described above plus 150 μl of antiserum which was added to 4.1 ml of warm agarose. (The antiserum volume must be determined empirically.) After thorough mixing, the antiserum-containing agarose was pipetted onto the slides containing the first-dimension gel strip, and the gels were allowed to stand in the cold for 20 min. A second sample well was punched in the first-dimension strip, approximately 1.5 cm from the opposite end of the strip, and a duplicate sample was applied. Comparison could then be made between the area of the rocket produced on migration of the sample directly into the antiserum gel and the area of the rocket produced after a prior electrophoresis in the first dimension. The plates were again placed on wicks, gel side down, and the electrophoresis was performed in the second dimension at 2.5 V/cm per slide for 18 hr. Electrophoresis in both dimensions was performed in the cold (6°C), and the apparatus was covered with Lucite covers to prevent evaporation from or condensation on the gel surface.

Following electrophoresis, the slides were rinsed in 0.1 M NaCl and in distilled water for 40 min each with two changes of solution in both cases. Each plate was then covered with filter paper and placed in a 60°C drying oven for 45–60 min to dry the agarose. Slides were stained in 0.05% Coomassie Brilliant Blue R in ethanol–acetic acid–water 45:10:45 for 20 min and destained in ethanol–acetic acid–water 45:10:45 until the background was destained (5–10 min). **Fig. 1** shows a typical slide.

The standard curve (**Fig. 2**) for the quantitation of the arginine-rich apoprotein was established using a monospecific antiserum and a purified antigen from rat VLDL. Protein concentrations in the samples used for the calibration curve were determined by the method of Lowry et al. (17). Samples having concentrations of 0.05, 0.10, 0.25, 0.4, and 0.5 mg/ml of arginine-rich apoprotein were incubated in 1% Triton for 2 hr at room temperature and two-

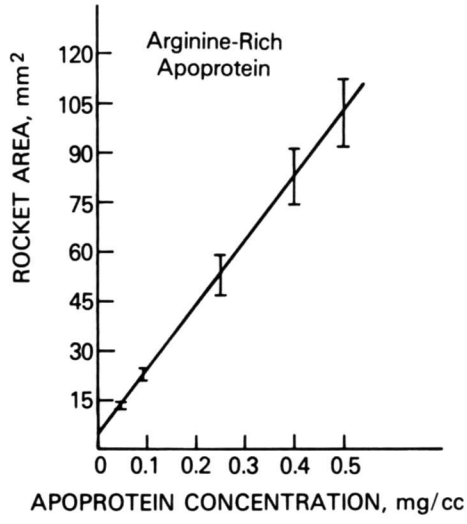

Fig. 2. Standard curve obtained by two-dimensional immunoelectrophoresis for the purified arginine-rich apoprotein using the monovalent antiserum to the arginine-rich apoprotein. The data represent 50 arginine-rich apoprotein standards analyzed during six different assays.

dimensional immunoelectrophoresis was performed as described above. For the standard curves, areas under rockets were plotted vs. known concentrations of pure apoprotein antigen and the data were fitted to a least-squares line by regression analysis. The standard deviation of the line (Σ_{yx}) was 7.2 (average of 50 data points; 6 different assays) with a correlation coefficient (r) of 0.977. An overview of the results indicated that the data fit well to the line, with scatter being approximately equal on both sides of the line. A one-way analysis of variance showed that the results obtained on duplicate samples run on the same set of slides (intraassay) varied 1.5–13%, with the largest variation occurring at the higher concentrations. Variation of the results obtained on different sets (interassay) was 3.3–13.5%. The good fit of these data and the agreement between duplicates in one assay or from assay to assay justified the assumption of linearity as well as the use of this standard curve over the range of concentrations shown in Fig. 2 to determine the apoprotein content of the lipoprotein samples. Areas under the rockets were calculated from height times width at half-height after measuring the height and width by use of a calibrated lens. This method was determined to be at least as accurate as any other method that was used (including planimetry and projection of the rockets on a photographic screen). Concentrations of unknown samples were then found from the standard curve.

Monovalent antisera to the arginine-rich apoprotein were prepared in New Zealand white rabbits. The antigen (200–500 μg) in 1 ml of 0.15 M NaCl was emulsified with an equal volume of Freund's complete adjuvant and injected at multiple sites intradermally. After two 3-week intervals, additional injections were given in the same manner, except that the antigen was emulsified in Freund's incomplete adjuvant. Serum was obtained 7–8 days after the second booster injection to determine the antiserum titer. Additional boosts were occasionally required. Antisera used in the immunoelectrophoretic procedure reacted only with the purified apoprotein and not with LDL or Triton-solubilized B apoprotein, the C apoprotein column fraction, apo-A-II, apo-A-I, or rat albumin.

Isolation of proteins for antisera production. Purified rat arginine-rich apoprotein for antisera production was prepared from hypercholesterolemic rat VLDL by gel chromatography. The apoproteins were solubilized in column buffer, 0.2 M Tris-Cl (pH 8.2) containing 4 M guanidine-HCl, and chromatographed on 300 × 2.5 cm G-200 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) columns equilibrated with column buffer. Fractions were dialyzed against 5 mM NH_4HCO_3 (pH 8.2) and lyophilized.

Validation of two-dimensional immunoelectrophoresis. The method was validated by comparison of the arginine-rich protein concentration obtained by two-dimensional immunoelectrophoresis on the native lipoproteins with the values obtained by column chromatography of control and hypercholesterolemic VLDL. Removal of the B apoprotein and delipidation of the lipoproteins were accomplished by treatment with tetramethylurea (24). The tetramethylurea-soluble protein was dialyzed against 5 mM NH_4HCO_3 (pH 8.2) and lyophilized. Residual lipid was removed by extraction with one part chloroform–methanol 2:1 and one part diethyl ether. The apoproteins were solubilized in column buffer, 0.2 M Tris-Cl (pH 8.2) containing 4 M guanidine-HCl, and chromatographed on G-200 Sephadex (Pharmacia), 300 × 2.5 cm, equilibrated with column buffer. Fractions were exhaustively dialyzed against 5 mM NH_4HCO_3 (pH 8.2) and protein determinations were done on lyophilized aliquots (17).

RESULTS

Detailed characterization of cholesterol-induced hyperlipoproteinemia

Rats fed laboratory chow supplemented with lard, cholesterol, taurocholate, and propylthiouracil (cholesterol diet) developed a variable hypercholesterolemia that ranged from approximately 300 to 600 mg/dl as compared to a plasma cholesterol of less than 80 mg/dl for control rats. The spectrum of plasma lipoproteins changed from a pattern of principally high density lipoproteins in control rats to a hyperlipoproteinemia characterized by an increase in β- and α_2-migrating lipoproteins and a decrease in α_1-migrating HDL following cholesterol feeding (**Fig. 3**). These changes will be shown to correspond to an increase in B-VLDL, LDL, and HDL_c lipoproteins and a decrease in the typical HDL.

An analysis of multiple ultracentrifugal density fractions obtained from the hypercholesterolemic rats by paper or agarose electrophoresis revealed the presence of several different lipoproteins based on electrophoretic mobility (Fig. 3). The $d < 1.006$ fraction contained a β-migrating lipoprotein referred to as B-VLDL and a pre-β-migrating VLDL. There was a β band (LDL) in the density fractions between 1.006 and 1.06 associated with an α-migrating band to be referred to as HDL_c. The prominence of the HDL_c in the density fraction 1.006–1.02 was directly correlated with the degree of hypercholesterolemia. The oc-

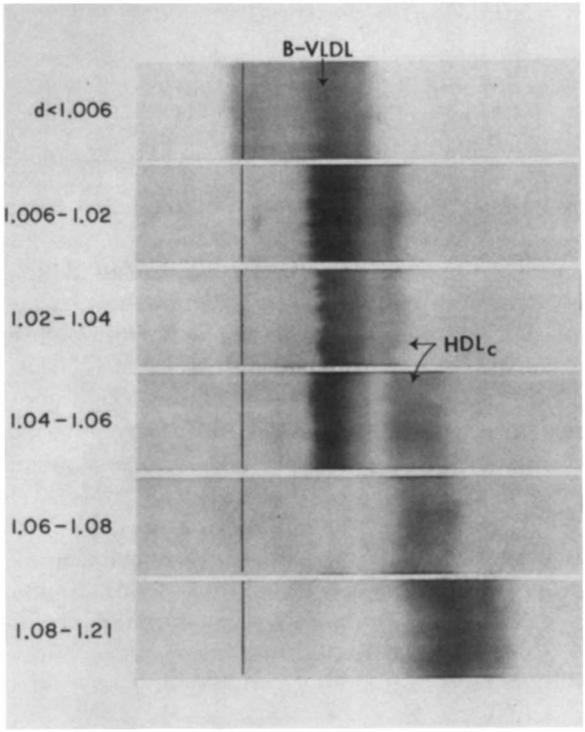

Fig. 3. Paper electrophoretograms of the lipoproteins in various ultracentrifugal density fractions for rats on the cholesterol diet.

curence of HDL_c, defined as an α-migrating lipoprotein in the low density range that lacked the B apoprotein, has been described following cholesterol feeding in dogs and swine (4, 5). The HDL_c in the d 1.06–1.08 fraction had α migration, slightly faster than that of the lower density HDL_c and slower than the typical HDL at higher density (1.08–1.21).

A comparison of the lipid and protein distributions of various ultracentrifugal density fractions from control and cholesterol-fed rats revealed that, following cholesterol feeding, the lipoproteins at lower densities were increased in prominence (**Table 1**). More than half of the plasma cholesterol and lipoprotein protein in control rats was transported by lipoproteins of $d > 1.08$, whereas following cholesterol feeding there was a marked shift to lipoproteins of lower density. The prominence of cholesterol-transporting lipoproteins in the $d < 1.006$ fraction as compared to the intermediate fraction (1.006–1.02) appeared to correlate directly with the degree of hypercholesterolemia as presented in Table 1. There was an absolute as well as a relative decrease in total HDL (d 1.08–1.21). Total lipoprotein protein recovered in the multiple density fractions revealed a 1.5- to 2-fold increase as a result of cholesterol feeding as compared to total lipoprotein protein in the plasma of control animals.

The ultracentrifugal density fractions that clearly contained two or more plasma lipoprotein classes were further fractionated by preparative Geon–Pevikon electrophoresis as previously reported for the purification of dog and swine lipoproteins (4, 5). The $d < 1.006$ fraction could be separated by the Geon–Pevikon electrophoretic procedure into two bands, one with β mobility (B-VLDL) (~6 cm from origin) and a second with pre-β mobility (VLDL) (~9 cm from origin). From the various ultracentrifugal fractions between density 1.006 and 1.08, the β-migrating lipoprotein referred to as LDL and the α-migrating lipoprotein (HDL_c) were isolated by the electrophoretic procedure. The LDL migrated ~6 cm and the HDL_c ~11 cm from the origin. The HDL that occurred at d 1.08–1.21 were not subjected to further fractionation procedures.

The chemical composition of the lipoproteins isolated as described above from a representative pool of plasma from cholesterol-fed rats is presented in **Table 2. Table 3** presents the particle sizes of these classes of lipoproteins, from the same plasma pool, as determined by negative staining electron microscopy. These results are similar to results obtained from three separate plasma pools subjected to identical procedures. LDL and HDL_c (Tables 2 and 3) were both cholesterol-rich (approximately 75% was esterified) and similar in size. However, as will be shown, they were distinctly different with respect to apoprotein content. The HDL (d 1.08–1.21) were protein- and phospholipid-rich lipoproteins with a parti-

TABLE 1. Percent distribution of lipid and protein among ultracentrifugal fractions

	Triglyceride	Total Cholesterol	Phospholipid	Protein
Control[a]				
Plasma (mg/dl)	70	58	110	
d < 1.006	97.5	14.6	29.8	9.8
1.006–1.04	1.2	5.2	4.4	5.4
1.04–1.08	1.1	26.1	16.7	15.0
1.08–1.21	0.2	54.0	49.0	69.8
Cholesterol-fed[b]				
Plasma (mg/dl)	194	680	488	
d < 1.006	98.5	84.6	82.6	66.3
1.006–1.04	0.5	12.0	12.0	12.8
1.04–1.08	0.5	0.5	0.9	2.0
1.08–1.21	0.5	2.9	4.5	18.9
Cholesterol-fed[b]				
Plasma (mg/dl)	52	259	171	
d < 1.006	95.8	54.2	47.8	18.0
1.006–1.02	1.8	24.8	24.2	20.6
1.02–1.04	2.2	10.5	11.3	12.8
1.04–1.08	0.1	3.7	4.6	9.2
1.08–1.21	0.1	6.9	12.1	39.3

[a] Data represent a composite from six pools of 12–20 rats each.
[b] Cholesterol diet. These two sets of data represent the extremes of plasma cholesterol. Each set of data was obtained from one pool of 12 rats.

TABLE 2. Percent composition of plasma lipoproteins from cholesterol-fed rats

	B-VLDL–VLDL	LDL	HDL_c	HDL
d < 1.006				
Triglyceride	14.3			
Total cholesterol	58.0			
Phospholipid	22.2			
Protein	5.2			
d 1.006–1.02				
Triglyceride		0.8	0.9	
Total cholesterol		59.0	65.7	
Phospholipid		29.9	20.7	
Protein		10.2	12.6	
d 1.02–1.04				
Triglyceride		0.5	1.2	
Total cholesterol		63.4	42.8	
Phospholipid		21.1	41.5	
Protein		14.8	14.2	
d 1.04–1.08				
Triglyceride		1.0	0.9	
Total cholesterol		52.9	36.7	
Phospholipid		28.3	43.1	
Protein		17.3	19.2	
d 1.08–1.21				
Triglyceride				0.1
Total cholesterol				28.1
Phospholipid				28.2
Protein				43.7

cle size of 80–120 Å in diameter. The composition (25) and size (26) of the HDL were similar to reported values for control plasma HDL.

Polyacrylamide gel electrophoresis of the several classes of lipoproteins revealed the marked prominence of a band that has been identified as the arginine-rich apoprotein (ARP) (**Fig. 4**). The ARP isolated on G-200 Sephadex in 4 M guanidine from the d < 1.006 fraction of cholesterol-fed rats had a molecular weight of approximately 34,000, contained ~11 mol percent arginine, and coelectrophoresed with the arginine-rich apoprotein of swine B-VLDL and HDL_c and human B-VLDL (5). Detailed characterization of the ARP, shown in Fig. 4 for comparison, will be presented elsewhere[1] (27). The A-I apoprotein isolated from control rat HDL, shown in Fig. 4, appears to be homologous to the A-I apoprotein of man (27). Rat A-I coelectrophoresed with the A-I apoprotein of dog, swine, Patas monkey, and man.[2] The apoprotein content of rat HDL has been reported (28).

In Fig. 4 it can be seen that several of the plasma lipoproteins from the cholesterol-fed rats contained a band that coelectrophoresed with the ARP (band b). The ARP was a major protein constituent of the d < 1.006 fraction, the intermediate lipoproteins (d 1.006–1.02), and the LDL and HDL_c. A striking difference between the gel patterns of the LDL and HDL_c was the apparent absence of the B apoprotein (band a) from the HDL_c. The lack of detectable B apoprotein was confirmed immunochemically by the observation that the HDL_c did not react with antisera to LDL. The HDL_c, particularly at the higher density (d 1.06–1.08), possessed a band that coelectrophoresed with the A-I apoprotein. The HDL (d 1.08–1.21) contained primarily the A-I apoprotein and the faster migrating C apoproteins. Unlike HDL from control rats, the HDL obtained after cholesterol feeding often did not contain the ARP. Several of the lipoproteins possessed slower migrating bands near the tops of the gels which have not been characterized. The lower molecular weight (C) apoproteins near the bottom of the gel were poorly resolved on the SDS–polyacrylamide gels but have previously been well characterized by Herbert et al. (29).

Quantitative two-dimensional immunoelectrophoresis

The two-dimensional immunoelectrophoretic procedure was based on the methodology of Laurell (22) and Davies (20). The immunoprecipitation of the antigen in the antibody-containing gel produced an arc that had the shape of a rounded triangle (the Laurell "rocket"). This was a clearly defined area with a base determined by the boundary between the first- and second-dimension gels. It has been shown previously that the area under the rocket is directly proportional to the concentration of antigen in the sample (For a review of this method, see reference 30.) Our work with purified antigens and monovalent antibodies confirmed this.

The use of Triton X-100 for solubilization and de-

TABLE 3. Particle size (Å) by negative staining[a]

	B-VLDL	VLDL	LDL	HDL_c	HDL
1.006	350–700 (450)	300–850 (500)			
1.02			300–400 (350)	250–450 (325)	
1.04			250–350 (310)	200–325 (275)	
1.08			185–300 (250)	125–200 (175)	
1.21					80–120 (100)

[a] The diameters of approximately 200 particles for each lipoprotein class were measured. Each range represents more than 95% of the particles measured excluding the occasional particles at either end of the spectrum of sizes. Data are presented as the range with the mean diameter in the parentheses.

[1] Weisgraber, K. H., R. W. Mahley, and G. Assman, manuscript in preparation.

[2] Mahley, R. W., unpublished observation.

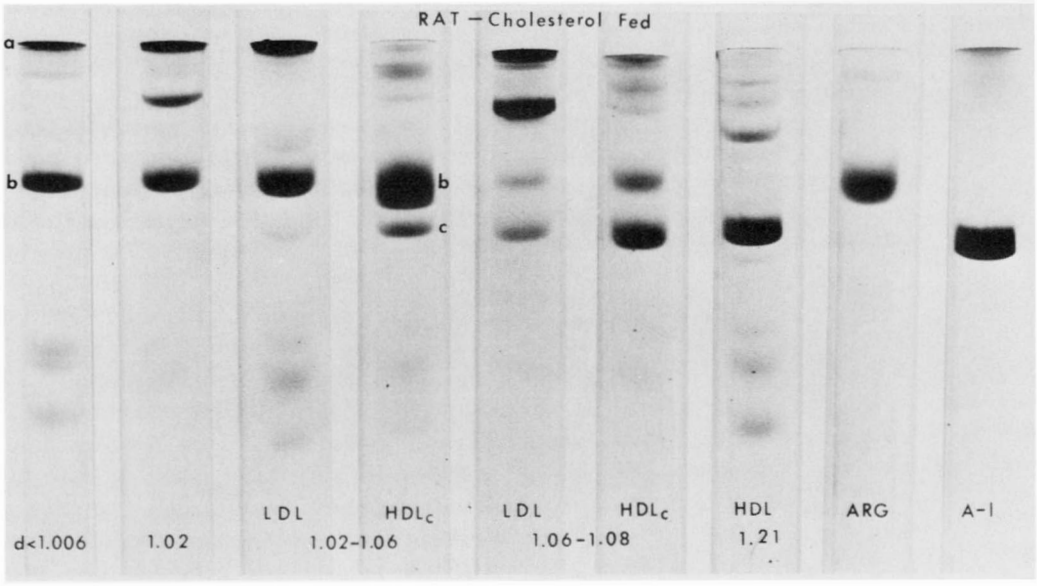

Fig. 4. Sodium dodecyl sulfate polyacrylamide gels of the apolipoproteins in the various ultracentrifugal density fractions from rats on the cholesterol diet. The LDL and HDL_c were purified by Geon–Pevikon electrophoresis prior to apoprotein analysis. Bands labeled a–c correspond to the B, arginine-rich (ARG), and A-I apoproteins. Approximately 15 μg of protein was applied.

lipidation of the lipoprotein samples was adapted from the work of Bjerrum and Lundahl (9). Incubation time and concentration of detergent were determined experimentally. In an attempt to improve resolution, dextran (average mol wt 73,000) was added to the buffer and gels (23). The presence of dextran did not affect the results except to improve the rocket resolution by more clearly defining the boundaries of the rockets. The buffer chambers contained barbital buffer with 0.05% Triton X-100 and 2% dextran, while the gels were composed of 1% agarose, 2% dextran, and 0.05% Triton X-100.

CHART I. Recovery of arginine-rich protein from rat VLDL on Sephadex column chromatography vs. two-dimensional immunoelectrophoresis (2-d IEP)

CONTROL RAT	HYPERCHOLESTEROLEMIC RAT
24 mg Total VLDL Protein	20.5 mg Total d < 1.006 Protein
2-d IEP	2-d IEP
15.7% ARP[b]	38.0% ARP[b]
TMU[a] delipidation	TMU delipidation
50% insoluble	30% insoluble
11.9 mg TMU-soluble protein	14.3 mg TMU-soluble protein
Sephadex Column[c] / 2-d IEP	Sephadex Column / 2-d IEP
29.6% ARP (14.8%)[d] / 28.6% ARP (14.3%)	52.8% (37.1%) / 55.8% (38.9%)

[a] Tetramethylurea.
[b] Percent of the total protein represented by the arginine-rich protein in the native lipoproteins.
[c] Sephadex G-200, eluted with 4 M guanidine.
[d] Conversion of the values back to the percent ARP in the native lipoprotein by taking into account the TMU insoluble (B) content.

Results for lipoprotein or plasma samples obtained by precisely the same procedures were reported as milligrams of the arginine-rich apoprotein per 100 ml of plasma. All results reported were obtained from samples subjected to electrophoresis in both dimensions. However, in most cases, an identical sample was applied after the first-dimension electrophoresis was completed (indicated by the arrow in Fig. 1). As can be appreciated, the sample subjected only to the second-dimension electrophoresis had a less distinct rocket, possibly caused by the presence of lipid in the region of the rocket. When slides from samples run in both dimensions were stained with Oil Red O, the lipid could be seen to remain at the origin away from the rocket. No protein-stainable or immunoprecipitable material remained at the origin or associated with the lipid.

Validation of two-dimensional immunoelectrophoretic procedure

The method of two-dimensional immunoelectrophoresis was validated by a comparison with the results obtained from Sephadex G-200 column chromatography (see Flow Chart I). The total ARP in the d < 1.006 (VLDL) fraction from a group of

control rats was determined by the two-dimensional immunoelectrophoretic procedure to be 15.7% of the total protein in the native lipoproteins. Delipidation with tetramethylurea resulted in solubilization of approximately 50% of the apoprotein including the arginine-rich apoprotein and the low molecular weight C apoproteins. Separate aliquots of the TMU-soluble proteins were subjected to Sephadex column chromatography for isolation of the ARP and to two-dimensional immunoelectrophoresis. The ARP represented 29.6% of the TMU-soluble proteins as determined by column chromatography and 28.6% as determined by two-dimensional immunoelectrophoresis. Conversion of the results determined on the TMU-soluble apoproteins back to the native lipoproteins showed that approximately 14–15% of the total VLDL protein was represented by the ARP. This is in good agreement with the 15.7% determined by immunoelectrophoresis for the original native lipoprotein sample.

As determined by two-dimensional immunoelectrophoresis, the ARP in the native d < 1.006 fraction from cholesterol-fed rats represented 38% of the total protein (Chart I). Approximately 30% of the protein (apo-B) remained insoluble following TMU delipidation. Of the TMU-soluble fraction, 52.8% of the protein applied to the Sephadex column was identified as the ARP as compared to 55.8% determined by two-dimensional immunoelectrophoresis. Conversion of these results to the percent of total protein represented by ARP in the native lipoproteins revealed that 37.1% and 38.9% of the total d < 1.006 protein was the ARP as determined by column chromatography and two-dimensional immunoelectrophoresis, respectively. These results are in good agreement with the 38% ARP determined in the native d < 1.006.

In an additional study, the arginine-rich apoprotein component of the TMU-soluble proteins from the d < 1.006 fraction from hypercholesterolemic rats was determined by column chromatography and by two-dimensional immunoelectrophoresis to be 38% and 36%, respectively.

Quantitation of the arginine-rich apoprotein in plasma and ultracentrifugal fractions

The total ARP was determined on the plasma of rats fed various control diets (as defined in **Table 4**). The mean total plasma ARP was approximately 29 mg/dl. When rats were fed the hypercholesterolemic diet (cholesterol, lard, propylthiouracil, and taurocholate), the ARP was significantly elevated to approximately 47 mg/dl (Table 4).

The distribution of the ARP in the four ultracentrifugal fractions isolated and characterized in the first part of this paper is presented in **Table 5**. In the control rats less than 10% of the ARP was found in the d < 1.006 fraction as compared to >50% in the d 1.063–1.21 fraction, which represented typical rat HDL. The remainder was in the d 1.02–1.063 fraction, which contained essentially all of the LDL and the low density HDL. The ARP represented 4–15% of the total VLDL (d < 1.006) protein, 0–6% of the total d 1.006–1.02 protein, 7–30% of the d 1.02–1.063 protein, and 8–15% of the total HDL (d 1.063–1.21) protein.

The distribution of the ARP in the ultracentrifugal fractions from the cholesterol-fed animals revealed a marked increase in this apoprotein in the d < 1.006 and d 1.006–1.02 and 1.02–1.063 fractions, as shown in Table 5. The ARP represented 21–46% of the total d < 1.006 protein (B-VLDL and VLDL), 29–58% of the total d 1.006–1.02 protein, and 20–51% of the total d 1.02–1.063 protein (LDL and HDL_c). This apoprotein was significantly reduced ($P < 0.02$) in the d 1.063–1.21 fraction, representing only 1–5% of the total HDL protein.

It is notable, however, that the total lipoprotein-associated ARP obtained by summing the results in the four ultracentrifugal fractions (Table 5) was markedly different from the total ARP determined

TABLE 4. Total plasma arginine-rich apoprotein

	Number	mg/dl	
Control I[a]	53	30.9 ± 6	N.S.[f]
Control II[b]	45	28.7 ± 8	N.S.
Control III[c]	35	29.5 ± 8	N.S.
Control IV[d]	24	29.8 ± 8	N.S.
Cholesterol diet[e]	37	47.2 ± 13	$P < .005$

[a] Rats fed commercial chow.
[b] Rats fed commercial chow plus 0.1% propylthiouracil.
[c] Rats fed commercial chow plus propylthiouracil and lard.
[d] Rats fed commercial chow plus cholesterol and lard.
[e] Rats fed commercial chow plus cholesterol, lard, propylthiouracil, and taurocholic acid.
[f] Not significant.

TABLE 5. Mean concentrations (mg/dl)[a] of the arginine-rich apoprotein in rat plasma lipoproteins isolated in the 60 Ti rotor

	Control[b]	Cholesterol-fed[c]
Total	6.2 ± 2.6	24.7 ± 7.3
d < 1.006	0.4 ± 0.26	5.2 ± 3.3
d 1.006–1.02	0.01 ± 0.02	4.8 ± 1.6
d 1.02–1.063	1.9 ± 1.4	17.2 ± 6.9
d 1.063–1.21	4.0 ± 1.6	0.77 ± 0.58

[a] Mean ± standard deviation.
[b] Means of determinations on six different pools (12 rats each) of control rat lipoproteins.
[c] Means of determinations on six different pools (12 rats each) of lipoproteins from animals fed cholesterol, lard, taurocholate, and propylthiouracil.

on whole plasma (Table 4). The total ARP in the four ultracentrifugal fractions of the control rats was 6.2 mg/dl as compared to a total plasma ARP of 29 mg/dl. In the cholesterol-fed rats, 24.7 mg/dl of ARP was recovered in the lipoprotein fractions (Table 5) as compared to 47 mg/dl in the plasma (Table 4). Therefore, less than 50% of the total plasma ARP was associated with the lipoproteins after the ultracentrifugal procedure. In the original set of experiments (Table 5) the ARP in the d > 1.21 ultracentrifugal fraction was not quantitated. However, subsequent experiments in which the d > 1.21 fraction was analyzed showed that at least 40% of the ARP could be measured in the d > 1.21 fraction when sequential ultracentrifugation with a 60 Ti rotor at 59,000 rpm was utilized. The effects of ultracentrifugation on the ARP content of lipoproteins were further investigated by comparing the results obtained by multiple sequential ultracentrifugation in the 60 Ti to those obtained by limited ultracentrifugation in the 40 rotor.

Three fractions (d < 1.006, d 1.006–1.21, and d > 1.21) from the plasma of control and cholesterol-fed animals were separated using a 40 rotor and the quantitative distribution of the plasma ARP was determined. When the results obtained with the 60 Ti rotor (Table 5) were compared to those obtained with the 40 rotor (**Table 6**), there was a 10-fold difference in the amount of ARP in the d < 1.006 fraction of control rats and a 3-fold difference in the ARP in the d < 1.006 fraction of the cholesterol-fed rats, with less ARP associated with the lipoproteins isolated with the 60 Ti rotor. Likewise, there was a significant loss of ARP from the control rat d 1.006–1.02, 1.02–1.063, and 1.063–1.21 fractions isolated with the 60 Ti rotor as compared to the ARP measured in the d 1.006–1.21 fraction obtained with the 40 rotor. However, in the cholesterol-fed rats the quantity of ARP in the d 1.006–1.02, 1.02–1.063, and 1.063–1.21 fractions obtained with the 60 Ti rotor (Table 5) was approximately equal to that obtained by the single ultracentrifugation (d 1.006–1.21) with the 40 rotor. The cholesterol-rich lipoproteins in the d 1.006–1.21 fractions from the cholesterol-fed rats may bind the ARP more strongly than the lipoproteins in these fractions in control rats. In control rats the majority of the ARP was associated with the typical HDL (d 1.063–1.21; Table 5), whereas in the cholesterol-fed rats the d 1.02–1.063 (LDL and HDL_c) contained most of the ARP. Thus, the use of the 60 Ti rotor caused marked quantitative changes in the ARP content of the rat plasma lipoproteins (a detailed description of alterations in rat and human lipoprotein ARP caused by ultracentrifugation will be presented elsewhere).

TABLE 6. Distribution of ARP following ultracentrifugation in a 40 rotor

Diet	(N)	d < 1.006[c]	1.006–1.21	>1.21	% Recovery
Control[a]	(6)	3.5 ± 0.9	22.7 ± 3	7.7 ± 2	87–96%
Cholesterol-fed[b]	(6)	17.5 ± 7	24.1 ± 3	4.6 ± 1	88–95%

[a] Commercial chow.
[b] Commercial chow plus cholesterol, lard, propylthiouracil and taurocholate.
[c] Isolation of the d < 1.006 and d 1.006–1.21 fractions using the 40 rotor at 39,000 rpm was accomplished by ultracentrifugation for 18 and 48 hr, respectively.

DISCUSSION

As a model for experimental atherosclerosis, the rat has severe limitations because of resistance to hypercholesterolemia and the small size of the arterial vessels, hindering histologic or chemical studies. However, with respect to lipoprotein metabolism, the rat is the most widely studied species, and much of the methodology designed to investigate lipoprotein metabolism (organ perfusion, organelle isolation, cell metabolism) is readily applied to these animals. For these reasons we have undertaken to characterize the dietary hyperlipoproteinemia of the rat as a basis for further studies of lipoprotein metabolism.

Cholesterol feeding in association with other dietary additives such as bile acids and propylthiouracil complicates the interpretation of the results. However, when using such resistant species as the rat and dog as models of atherosclerosis, such extremes in dietary protocol are required to induce significant hypercholesterolemia. On the other hand, animal models for atherosclerosis such as the miniature swine and rabbit readily develop hypercholesterolemia with only the addition of fat and cholesterol to the diet. Despite differences in the diets, there do appear to be in all these lower species certain consistent features of the hyperlipoproteinemia associated with the development of atherosclerosis, including: (*a*) the presence of B-VLDL similar in many respects to the β-migrating lipoprotein in the d < 1.006 fraction of Type III patients (7), (*b*) an increase in the intermediate lipoproteins considered by some to represent remnants of chylomicron and VLDL catabolism (31–35), (*c*) an increase in the LDL, and (*d*) the appearance of a lipoprotein referred to as HDL_c, which becomes a prominent cholesterol-carrying lipoprotein. Associated with all of these cholesterol-induced lipoproteins is a prominence of a particular apo-

protein that has been identified as the arginine-rich apoprotein (4, 5). The origin, fate, and role of this apoprotein in the cholesterol-fed animals are open to speculation.

In control rats, the principal plasma lipoproteins are the HDL (d 1.063–1.21 and extending down to d 1.04), with a very low concentration of the lower density lipoproteins. Following cholesterol feeding, the resulting hyperlipoproteinemia is characterized by the features outlined above, with the lipoproteins in the low and very low density fractions becoming the principal cholesterol-transporting lipoproteins. The LDL and HDL_c induced by cholesterol feeding are both cholesterol-rich lipoproteins (40–60% of their total composition), have approximately the same particle size, and overlap in density (~d 1.02–1.06). However, they differ in that the HDL_c lack the B apoprotein and contain predominantly the ARP and a variable amount of the A-I and C apoproteins. The B-VLDL, intermediate, and LDL also contain the ARP. The typical HDL (d 1.08–1.21 or 1.063–1.21) are relatively and absolutely reduced after cholesterol feeding and qualitatively appear to have a reduced amount of the ARP. Reduction in HDL following cholesterol feeding has been reported in several species (4, 36–38).

These qualitative impressions as to the altered distribution of the ARP prompted us to establish an immunochemical procedure for quantitation of this rat apolipoprotein. The results obtained by the two-dimensional quantitative immunoelectrophoretic procedure demonstrate that the total plasma ARP is increased following the cholesterol diet. However, these studies reveal the unexpected result that the ARP is readily removed from rat lipoproteins by ultracentrifugation, particularly when the 60 Ti rotor is used. More than 50% of the total plasma ARP is unassociated with the lipoproteins following sequential ultracentrifugation using the 60 Ti rotor as described in this paper. When the 40 rotor is used to isolate the d < 1.006 and d 1.006–1.21 fractions, 10 and 20% of the total ARP are found in the d > 1.21 fraction of cholesterol-fed and control rats, respectively. Similar results have been found using human lipoproteins.[2] The quantity of the ARP that is present free in the plasma or at least unassociated with lipoproteins of d < 1.21 remains to be determined. The arginine-rich apoprotein has been described as free in lymph (39). Despite the quantitative changes induced by the ultracentrifugal procedures used, the qualitative impressions of the effects of cholesterol feeding on rat plasma lipoproteins remain valid. There is an increase in the ARP which is associated primarily with the d < 1.006 fraction, the intermediate fraction, and HDL_c. New procedures for the isolation of lipoproteins without ultracentrifugation are necessary to overcome the alterations in the lipoproteins and the losses of the ARP.

One objection to the use of quantitative immunochemical procedures with lipoproteins has been the inability to assay plasma samples that contain large and poorly diffusing or migrating lipoproteins such as large VLDL or chylomicrons. Another question was the possibility of masked antigenicity, which could result if the apoproteins were buried in the hydrophobic regions of the particles, thus excluding the antisera. Therefore, we attempted to develop a procedure by which lipoproteins were at least partially delipidated and the apoproteins were solubilized immediately prior to the immunochemical analysis. Various reagents including sodium dodecyl sulfate, Tween 20, and urea were unsuccessfully used. However, it was found that the use of the nonionic detergent Triton X-100 resulted in delipidation of the rat lipoproteins and, at low concentration, did not interfere with the formation of a quantitative immunoprecipitin reaction. Triton X-100 has been used successfully in the delipidation, solubilization, and immunochemical determination of various membrane proteins by procedures similar to those described for apolipoprotein determinations (8, 9). This methodology should be applicable to the analysis of other apoproteins and may serve as an additional analytical tool to study the metabolic consequences of high cholesterol diets.

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