Quantitation of human apolipoprotein C-III and its subspecies by radioimmunoassay and analytical isoelectric focusing: abnormal plasma triglyceride-rich lipoprotein apolipoprotein C-III subspecie concentrations in hypertriglyceridemia

M. L. Kashyap, L. S. Srivastava, B. A. Hynd, P. S. Gartside, and G. Perisutti

Department of Internal Medicine, University of Cincinnati Medical Center, Cincinnati, OH 45267

Abstract A specific, accurate, and sensitive double antibody radioimmunoassay for measuring human apolipoprotein (apo) C-III has been developed. Anti-apoC-III_1 developed in rabbits cross-reacted completely with apoC-III subspecies. Analytical isoelectric focusing of delipidated triglyceride-rich lipoproteins (TRL) was done to assess the percentage of total apoC-III mass comprised by apoC-III_0, C-III_1, and C-III_2, and the data were used to compute the absolute plasma TRL apoC-III subspecie concentration. Total plasma apoC-III was 11.1 ± 0.9 mg/dl (mean ± SEM) in 29 normolipidemic healthy subjects; 21.3 ± 4.9, 27.5 ± 2.2, and 53.6 ± 7 mg/dl in 3, 16, and 13 patients with primary types III, IV, and V hyperlipoproteinemia, respectively, and significantly ($P < 0.01$) higher than normal. Total plasma triglycerides (TG) correlated positively with total plasma apoC-III ($r = 0.88$; $P = 0.0001$) and TRL apoC-III ($r = 0.88$; $P = 0.0001$). Progressive hypertriglyceridemia was associated with a rise in the percent of total apoC-III in TRL isolated at $d < 1.006$ g/ml ($r = 0.78$; $P < 0.0001$; $n = 43$) and a reciprocal decline in the TRL-free plasma fraction ($d > 1.006$ g/ml). ApoC-III comprised significantly more of HDL_2 than HDL_3 protein (7.3 ± 0.2 versus 1.6 ± 0.2%, respectively, $P < 0.01$). HDL_2 and HDL_3 isolated from patients with type IV hyperlipoproteinemia had subnormal apoC-III as percent of total protein (2.4 ± 0.5 and 0.6 ± 0.1, respectively). Total plasma TG correlated negatively with *i*) apoC-III as percent of total HDL protein ($r = -0.67$; $P = 0.002$, $n = 20$); *ii*) apoC-III as percent of total HDL_2 protein ($r = -0.52$; $P = 0.019$); and *iii*) apoC-III as percent of total HDL_3 protein ($r = -0.72$; $P = 0.0004$). Plasma TRL apoC-III subspecie concentrations were significantly higher in the three hypertriglyceridemic groups (primary types III, IV, and V) compared to normals. TRL apoC-III_0 levels in patients with type IV and V were comparable (2.4 ± 0.3 and 2.2 ± 0.6 mg/dl, respectively). However, TRL apoC-III_1 and C-III_2 in patients with type V hyperlipoproteinemia were significantly higher ($P < 0.01$) than in patients with types IV or III hyperlipoproteinemia. Total plasma TG correlated positively with TRL apoC-III_0 ($r = 0.56$; $P = 0.0004$), TRL apoC-III_1 ($r = 0.82$; $P = 0.0001$) and TRL apoC-III_2 ($r = 0.76$; $P = 0.0001$). The slope of regression line relating total plasma TG with TRL apoC-III_1 was significantly steeper ($P < 0.0001$) than that for apoC-III_0. Thus, for a given interval of plasma TG, the change in concentration of TRL apoC-III_1 was much greater than that in TRL apoC-III_0. The development of the RIA and its combined use with analytical isoelectric focusing thus allows quantitation of this important glycopeptide and its subspecies in human plasma and its subfractions. Because apoC-III inhibits not only tissue lipoprotein lipase but also the hepatic uptake of triglyceride-rich lipoproteins and remnants, the data support the possibility that an abnormal metabolism of apoC-III subspecies may be linked pathogenetically to elevated plasma TG levels.—**Kashyap, M. L., L. S. Srivastava, B. A. Hynd, P. S. Gartside, and G. Perisutti.** Quantitation of human apolipoprotein C-III and its subspecies by radioimmunoassay and analytical isoelectric focusing: abnormal plasma triglyceride-rich lipoprotein apolipoprotein C-III subspecie concentrations in hypertriglyceridemia. *J. Lipid Res.* 1981. **22:** 800–810.

Supplementary key words lipoproteins · triglycerides · high density lipoproteins

The protein moiety of human lipoproteins is a heterogeneous mixture of numerous peptides grouped as apolipoproteins A, B, C, and so on. The C group of apolipoproteins is mainly found in the triglyceride-rich lipoproteins (i.e., very low density lipoproteins and chylomicrons) and high density lipoproteins (HDL). ApoC consists of three peptides (apoC-I, C-II, and C-III) with distinct differences in structure and physico-chemical properties (1). ApoC-III is a peptide (mol wt 9300) that exists in at least three forms. The species contain 0, 1, or 2 moles of sialic

Abbreviations: TRL, triglyceride-rich lipoprotein; TG, triglyceride; RIA, radioimmunoassay; IEF, isoelectric focusing; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; VHDL, very high density lipoprotein.

acid (2) and are known as apoC-III_0, C-III_1, and C-III_2, respectively.

Hypertriglyceridemia arising from normal fat digestion and absorption (resulting in chylomicronemia) or from endogenous triglyceride synthesis (resulting in increased levels of very low density lipoproteins) is associated with a transfer of the C apolipoproteins from HDL to TRL (3). The catabolic fate of TRL is mediated initially by lipoprotein lipase. In vitro studies have shown that apoC-II increases the activity of this enzyme (4–6) whereas apoC-III inhibits it (7).

Recent evidence with rat, canine, and human lipoproteins or with artificial lipid-protein complexes indicates that the C apolipoproteins may have a very important function in the uptake of TRL particles and remnants by the rat perfused liver. These studies suggest that whereas apoE or its isomers (E_3 and E_4) promote the recognition of TRL particles or remnants by a specific hepatic receptor, the C apolipoproteins have an opposite effect (8–12). Human apoC-III_1 has specifically been shown to inhibit this process (8). However, the exact in vivo role of apoC-III in normal human physiology has not been established.

The purpose of this report is to detail a double antibody radioimmunoassay we have developed for the quantitation of human apoC-III in plasma or its lipoprotein fractions. Analytical isoelectric focusing has been used to measure the proportions of the desialylated (C-III_0) and each sialylated (C-III_1 and C-III_2) apolipoprotein in delipidated TRL. By measuring total apoC-III by RIA and its subspecies by analytical IEF, the absolute concentrations of TRL apoC-III_0, C-III_1 and C-III_2 have been measured. Their relationship to total plasma triglycerides has been assessed. Additional work related to the amount of radioimmunoassayable apoC-III in HDL and its subfractions HDL_2 and HDL_3 and its relationship to plasma triglycerides is also presented.

METHODS

Radioimmunoassay of apoC-III

The design of the method developed in this laboratory was similar to an earlier one for human apoC-II (13).

Antigen isolation and characterization. The antigen used was apolipoprotein C-III_1 which was isolated from pooled human plasma. Blood was collected from fasting (>12 hr) asymptomatic normal individuals or fasting patients with type V hypertriglyceridemia in tubes containing the disodium salt of ethylenediaminetetraacetic acid (EDTA) to give a final concentration of 1.0 mg/ml. Plasma was separated by slow speed centrifugation at 4°C. VLDL was isolated by preparative ultracentrifugation for at least 18 hr in cellulose nitrate tubes placed in a 40.3 Beckman rotor (using a L5-50 Beckman Ultracentrifuge) according to Havel, Eder, and Bragdon (14). The VLDL was ultracentrifuged again after layering with 0.15 M sodium chloride in 1 mM disodium salt of EDTA (EDTA–saline) for further purification. ApoC-III_1 was isolated from the delipidated VLDL according to Brown, Levy, and Fredrickson (15). This method involved extensive dialysis of VLDL against EDTA–saline, its lyophilization and delipidation with cold (4°C) ethanol–diethyl ether at least thrice, followed by two washes with diethyl ether alone. The ethyl ether was evaporated under a gentle stream of nitrogen. The apoVLDL was dissolved in a 0.2 M Tris–HCl buffer, pH 8.2, containing 0.1 M sodium decyl sulfate. Approximately 40–60 mg of apoVLDL were applied to a G-150 Sephadex column (1.5 cm × 100 cm). Two major peaks of apoB and C were obtained. The C apolipoproteins were isolated, dialyzed extensively (using Spectrapor dialysis tubing with a molecular weight cut-off of 3,500) against 5 mM Tris–HCl, pH 8.2, at 4°C. This material was applied to a column of DEAE-cellulose equilibrated with 5 mM Tris–HCl, pH 8.2, at 4°C. Individual C apolipoproteins were separated by a sodium chloride gradient using a starting concentration of 0.05 M Tris–HCl, pH 8.2, at 4°C, and gradually increasing the sodium chloride concentration to 0.15 M. The total volume of the gradient was 500 ml. ApoC-I, C-II, C-III_1 and C-III_2 were resolved. The protein in tubes containing apoC-III_1 was subjected to analytical IEF (described below) for assessment of purity. The tubes from the top and descending slope of the apoC-III_1 peak, which showed a single band on analytical IEF, were pooled and dialyzed extensively against 0.05 M ammonium bicarbonate and lyophilized using a Virtis Uni-trap Model 10-100 lyophilizer (Virtis Ltd., Gardiner, NY 12525). Amino acid composition of the protein was determined with a Durrum-D-500 amino acid analyzer (Durrum Instrument Corp., Sunnyvale, CA). The amino acid composition was similar to published values based on the primary structure of apoC-III (2); purity was judged by analytical IEF (described below) and polyacrylamide gel electrophoresis in urea (16) and sodium dodecyl sulfate (17). Application of 100 μg of the protein per gel gave a single band by these methods. The pI for apoC-III_1 was 4.64 (19). **Fig. 1** shows the results of analytical IEF of 100 μg of this protein.

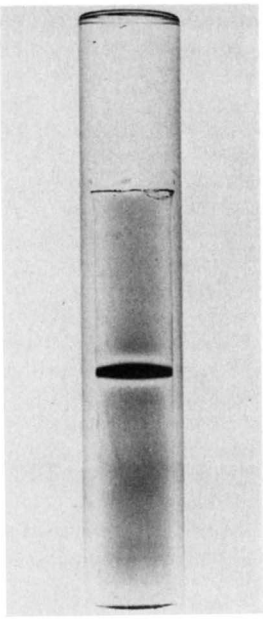

Fig. 1. ApoC-III$_1$ was isolated from delipidated triglyceride-rich lipoproteins isolated by preparative ultracentrifugation as detailed in Methods. Analytical IEF was carried out with 100 μg of protein.

Antibody preparation. Female albino New Zealand rabbits weighing approximately 2.5 kg were immunized with 100 μg apoC-III$_1$ (in 0.5 ml of 0.15 M sodium chloride) emulsified with 0.15 ml of Freund's complete adjuvant. Injections were given directly into the popliteal lymph nodes and also intradermally in multiple sites on the back. Booster injections (100 μg in Freund's complete adjuvant) on the back were given at approximately 3-week intervals, and the animals were bled 14 days after the last injection. The serum was kept at −70°C. Whole antiserum from one rabbit was used in this study. A dilution of 1:3,000 was used for the standard curve.

Labeling apoC-III$_1$ with ^{125}I. Basically, the method of Greenwood, Hunter, and Glover (18) was used. To 5 μg of lyophilized apoC-III$_1$, 0.1 ml of 0.5 M phosphate buffer, pH 7.6, was added and the tube was gently tapped to dissolve the apoprotein. This was followed by the addition of 0.5 mCi ^{125}I and 87.5 μg of chloramine-T in 25 μl of 0.05 M phosphate buffer, pH 7.6. After 30 sec, 250 μg of sodium metabisulfite in 0.1 ml of 0.05 M phosphate buffer, pH 7.6, was added to stop the reaction. Fifty μl of 7% bovine serum albumin was then added. The mixture was subjected to gel chromatography using Sephadex G-100 (1 cm × 100 cm column previously coated with 3% bovine serum albumin) and then eluted with 0.07 M barbital buffer containing 1% bovine serum albumin, pH 8.6, to separate labeled apoC-III$_1$ from ^{125}iodine. The elution profile showed an initial small peak of high molecular weight aggregates and a second larger peak of highly purified labeled apoC-III$_1$. The following procedures were used to assess homogeneity of the labeled apoC-III$_1$. When mixed with unlabeled apoC-III$_1$, both eluted in the same peak on gel chromatography and were noted as a single band on polyacrylamide gel electrophoresis (16). Thin gel slices subjected to scintillation counting also revealed a single peak that corresponded to the band stained with Coomassie Blue. The percent binding of labeled protein from the first and second peaks with initial antibody dilution of 1:3000 was 9% and 55%, respectively.

The assay. To a 10 × 75 mm glass tube were added *a*) 0.4 ml of 1% bovine serum albumin in 0.02 M barbital buffer (pH 8.5), *b*) 0.1 ml of plasma or unknown sample diluted 1:100–1:1200 (or greater) with barbital buffer, or apoC-III$_1$ standard, *c*) 0.1 ml of antibody diluted 1:10,000 with barbital buffer containing normal rabbit serum, and *d*) 0.1 ml of ^{125}I-labeled apoC-III$_1$ containing approximately 10,000 cpm. The assay was done with tubes at 4°C. After incubation of the mixture for 72 hr at 4°C, 0.1 ml of goat anti-rabbit gamma globulin was added. The samples were incubated for 48 hr at 4°C, and then centrifuged at 2,500 rpm for 20 min at 4°C. The supernate was aspirated and radioactivity in the washed (with 1.0 ml barbital buffer) precipitate was measured in a Packard Autogamma scintillation spectrometer 5230 (Packard Instrument Co., Inc., Downers Grove, IL). All measurements were done at least in duplicate and the mean concentration was used in the results.

Analytical isoelectric focusing of apoC-III in triglyceride-rich lipoproteins

The procedure followed for the determination of the ratios of apoC-III$_0$, C-III$_1$, and C-III$_2$ by analytical IEF was identical to that described and validated in detail by Kashyap et al. (19). Essentially, TRL isolated by preparative ultracentrifugation was delipidated by tetramethylurea and the apoC-III subspecies and apoC-II were separated by IEF in gels with a pI range between 3.5 and 5.0. This method clearly resolved the desialylated (apoC-III$_0$) and sialylated (apoC-III$_1$ and C-III$_2$) subspecies of apoC-III. Densitometric scanning of the gels was used for calculation of the percentage of total TRL apoC-III comprised by each apoC-III subspecies.

Other analytical methods

Total plasma triglycerides, cholesterol, HDL-cholesterol, and lipoprotein electrophoresis were done according to the Lipid Research Clinic Methodology

(20). Protein measurements were done by the method of Lowry et al. (21). For VLDL, the sample turbidity was removed by a modification of the assay using Triton X-100 as described by Kashyap, Hynd, and Robinson (22). Sequential density ultracentrifugation was performed according to the method of Havel et al. (14) for isolation of TRL (d < 1.006 g/ml), HDL_2 (d 1.063–1.125 g/ml), HDL_3 (d 1.125–1.210 g/ml), and very high density lipoproteins (VHDL) (d 1.210–1.250 g/ml). LDL used for cross-reactivity experiments was isolated from a normal healthy individual between a density interval of 1.030–1.050 g/ml. Delipidation of TRL was done according to Kane (23) using tetramethylurea. Incubation with neuraminidase was done according to a modification of the method of Morell et al. (24) and is described in detail elsewhere (19). Heating of plasma in experiments dealing with accuracy of the method was done at 52°C for 3 hr as described by Karlin et al. (25).

Study subjects

Studies were carried out on 29 normal healthy volunteers and 32 patients with primary hyperlipoproteinemias who were classified according to Lipid Research Clinics criteria (20). Mean (±SEM) age of the normal subjects was 33 ± 4 years. There were 3, 16, and 13 subjects with primary types III, IV, and V lipoprotein phenotypes whose ages were 33 ± 4, 50 ± 2, and 56 ± 3 years, respectively. Total plasma cholesterol was 195 ± 16 mg/dl in normals and 514 ± 92, 237 ± 10, and 414 ± 62 mg/dl in subjects with types III, IV, and V hyperlipoproteinemia, respectively; total plasma TG in normals was 114 ± 17 mg/dl and 576 ± 113, 498 ± 48, and 1646 ± 249 mg/dl, respectively, in the patients. Males comprised 23/29 normals, 3/3 type III, 13/16 type IV, and 5/13 type V individuals. At the time of study, all patients had been prescribed a diet appropriate for their hyperlipoproteinemia. All hypolipidemic drugs were discontinued for at least 6 weeks prior to this study. All patients fasted except for water for at least 12 hr. Venous blood was drawn in tubes containing EDTA to give a concentration of 1 mg/ml of blood. After centrifugation, the plasma was refrigerated and the determination of plasma lipids, electrophoresis, and preparative ultracentrifugation was begun as soon as possible. The distribution of plasma apoC-III in different lipoprotein subfractions (e.g., TRL or TRL-free plasma) was performed in a subset of 11 of the 29 subjects.

Statistical analyses

Means and standard errors were computed after first scrutinizing the data for discrepant values and gross errors. Variables with skewed distributions were then transformed using the logarithmic (base 10) function. Correlation coefficients, regression lines, differences among regression slopes, and Student's *t*-tests for differences between group means were computed by the standard methods as per Snedecor and Cochran (26).

RESULTS

The radioimmunoassay

Characterization of the anti-serum. Initial antibody dilutions of 1:3,000 were used for the assay. At this dilution, a typical standard curve (as previously described for apoC-II (13)) was observed (**Fig. 2**). Nonspecific binding to the tubes was <3% (range 0.5%–3.0%).

Cross-reactivity to apolipoproteins was measured as described previously (13). The percent cross-reactivity to anti-apoC-III_1 by apolipoproteins C-I, C-II, C-III_2, A-I, A-II, LDL protein, and d > 1.250 g/ml plasma fraction was 3.1, 1.0, 100.0, 0.16, 0.01, 1.35, and 0.01%, respectively (Fig. 2). The purity of apolipoproteins A and C was assessed by analytical IEF (19), polyacrylamide gel electrophoresis (16, 23) of 50 μg of protein, and amino acid analysis. The LDL was obtained by preparative ultracentrifugation using a narrow density range of 1.030–1.050 g/ml. A sample of this LDL was treated with tetramethylurea and the tube was centrifuged to precipitate apoB (16, 23). The supernate was subjected to analytical IEF and no band was noted after fixing and staining. As reported previously (19), the sensitivity of analytical IEF and polyacrylamide gel electrophoresis is approximately 2–3 μg of protein. Thus, the apolipoproteins were at least 94–96% pure. Cross-reactivity to apoC-III_0 was assessed by treating VLDL with neuraminidase as previously described (19). A TRL sample was incubated with neuraminidase and after 24 hr TRL was reisolated by ultracentrifugation. A control sample was incubated only with buffer without enzyme. The concentrations of apoC-III were found to be 664 and 660 μg/ml in the control and neuraminidase-treated sample, respectively. Analytical IEF of this sample showed a marked increase in the apoC-III_0 band, total absence of the apoC-III_2 band, and 80% reduction of apoC-III_1.

Serial dilutions of whole plasma, VLDL, intermediate density lipoproteins (d 1.006–1.019 g/ml), HDL_2, and HDL_3 fractions showed displacement curves that were parallel to the standard curve using apoC-III_1.

The sensitivity of the system was 0.8 ng apoC-III_1 per tube. For the standard curve, the working range

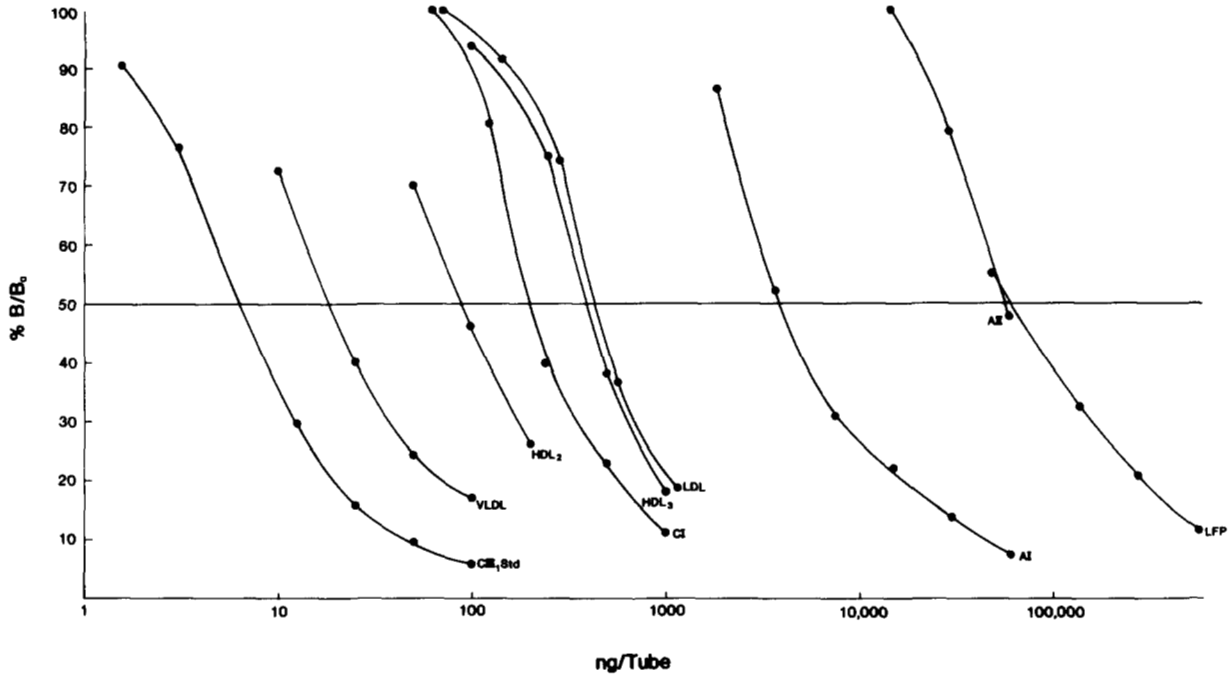

Fig. 2. Displacement of tracer (^{125}I-labeled apoC-III$_1$) by unlabeled apoC-III$_1$, apolipoproteins A-I, A-II, C-I, and C-II,LDL (apoB), and lipoprotein-free plasma.

was 1.56 to 100 ng. The inter- and intra-assay coefficient of variation was 9% and 7%, respectively.

The following experiments were conducted to assess the accuracy of the assay. *A*) Eighteen plasma samples from a group of subjects with a wide range in total plasma TG (50–2090 mg/dl) and apoC-III (38–507 μg/ml) were ultracentrifuged at d 1.006 g/ml. The d < 1.006 g/ml fraction (TRL) and the d > 1.006 g/ml fraction were analyzed for apoC-III. The sum of the concentrations in the two ultracentrifugal fractions (237.3 ± 30.8 μg/ml) was similar to the concentration in whole plasma (240.2 ± 32.0 μg/ml). The correlation coefficient (r) between the total plasma concentration and the sum of the two fractions was 0.99, $P < 0.001$.

B) To 0.5 ml of six different plasma samples, 20 μg of apoC-III, (dissolved in 0.15 M sodium chloride) was added. The volume of the plasma was brought to 1.0 ml with normal saline. One-half ml of 0.15 M saline was added to control tubes. Mean (±SEM) level of the samples to which apoC-III$_1$ was added was 153 ± 30 μg/ml; mean apoC-III concentration in the control sample was 132 ± 31 μg/ml. The correlation coefficient (r) between control and apoC-III$_1$ 'spiked' samples was 0.99; $P < 0.001$.

C) ApoC-III$_1$ isolated from delipidated TRL by chromatography (as described under Methods) in amounts of 0–40 μg was subjected to IEF in duplicate. After destaining, the apoC-III$_1$ band was scanned and the area under the peak was measured by planimetry. A standard curve relating the absolute amount of apoC-III$_1$ applied to its densitometric area was constructed. IEF of tetramethylurea-delipidated TRL from two subjects was performed in the same run as that of the standard curve. After destaining and scanning of the gels, the total area given by the apoC-III$_0$, C-III$_1$, and C-III$_2$ bands was used for quantitation of TRL apoC-III from the standard curve. The results by this method gave the values of 66.0 and 101.2 mg/dl; corresponding radioimmunoassay values were 68.1 and 106.0 mg/dl, respectively. In these two subjects the percent of total TRL protein as apoC-III was similar (33.7% and 40.2% by IEF versus 35.1 and 42.1% by RIA, respectively).

D) Seventeen plasma samples were heated at 52°C for 3 hr. Each sample was diluted with barbital buffer and subjected to RIA. Tubes containing control plasma were left at 4°C for the same period. Mean (±SEM) concentration of unheated control plasma was 111.6 ± 9.7 versus 112.4 ± 8.9 mg/dl for the preheated plasma. The correlation coefficient (r) between the apoC-III concentration of the preheated and control sample was 0.99; $P < 0.001$.

Distribution of apolipoprotein C-III in human plasma fractions; relationship to total plasma TG

Multiple regression analyses were done on the plasma measurements using age, sex, and body mass

index (weight/height^2) as independent variables in the normal subjects. No significant associations were found, i.e., age, sex or body mass did not influence the measurements in these subjects.

Total plasma apoC-III. **Fig. 3** shows the correlation between the total plasma TG and total plasma apoC-III over a wide range of TG (47–3860 mg/dl). In 43 subjects the correlation was 0.88 and was highly significant, $P = 0.0001$. The means ± SEM of the TG and apoC-III in 29 normal subjects were 114 ± 17 mg/dl and 11.1 ± 0.9 mg/dl, respectively; in 3 patients with primary type III hyperlipoproteinemia, the values were 576 ± 113 mg/dl and 21.3 ± 4.9 mg/dl, respectively; in 16 patients with type IV hyperlipoproteinemia, they were 498 ± 48 mg/dl and 27.5 ± 2.2 mg/dl, respectively; and in 13 primary type V, the concentrations were 1646 ± 249 mg/dl and 53.6 ± 7.0 mg/dl, respectively.

Total plasma TRL apoC-III. Total TRL apoC-III was calculated by subtracting apoC-III in the d > 1.006 g/ml infranate from the total plasma apoC-III. A highly significant correlation ($r = 0.88$; $P < 0.0001$) was observed between total plasma TG and TRL apoC-III (**Fig. 4**). The concentrations (mean ± SEM) of TRL apoC-III in normals (n = 11), primary types III (n = 3), IV (n = 16), and type V hyperlipoproteinemia (n = 13) were 2.8 ± 0.7, 12.7 ± 4.5, 18.8 ± 2.4, and 45.6 ± 6.6 mg/dl, respectively.

Distribution of plasma apoC-III in TRL and TRL-free plasma. **Fig. 5** shows the positive correlation between total plasma TG and the percent of total plasma apoC-III transported by TRL ($r = 0.78$; $P = 0.0001$). In 11 normals, and 3 type III, 16 type IV, and 13 type V patients, this percentage was 26.4 ± 0.6%, 55.7 ± 10.9%, 65.3 ± 5.6%, and 84.7 ± 2.4% respectively.

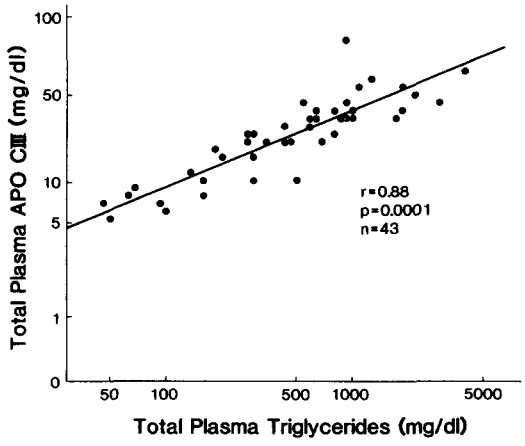

Fig. 3. The relationship between total plasma triglycerides and apoC-III by radioimmunoassay.

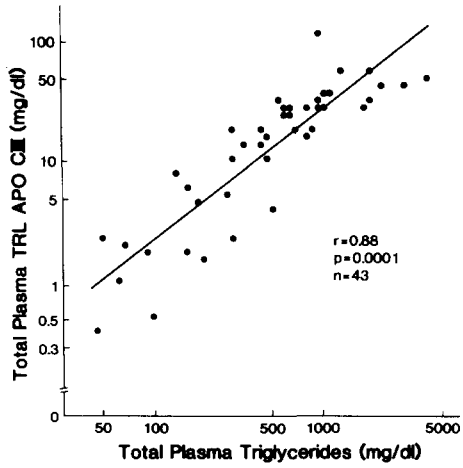

Fig. 4. The relationship between total plasma triglycerides and triglyceride-rich lipoprotein (TRL) total apoC-III by radioimmunoassay.

ApoC-III in HDL_2 and HDL_3. HDL_2 and HDL_3 were isolated in 20 subjects by preparative ultracentrifugation at density intervals between 1.063–1.125 g/ml, and 1.125–1.210 g/ml, respectively. Both subfractions of HDL were washed once by recentrifugation with sodium chloride–potassium bromide mixture at d 1.125 g/ml and d 1.210 g/ml, respectively, dialyzed against 0.15 M sodium chloride, and analyzed for protein. ApoC-III was measured by radioimmunoassay. Plasma samples from 21 subjects were obtained for this study. There were 16 normolipidemic subjects and 5 patients with type IV hyperlipoproteinemia. In two normal subjects, only HDL_2 or HDL_3 was isolated. Thus, complete data for both HDL_2 and HDL_3 were obtained only in 19 individuals. **Table 1** sum-

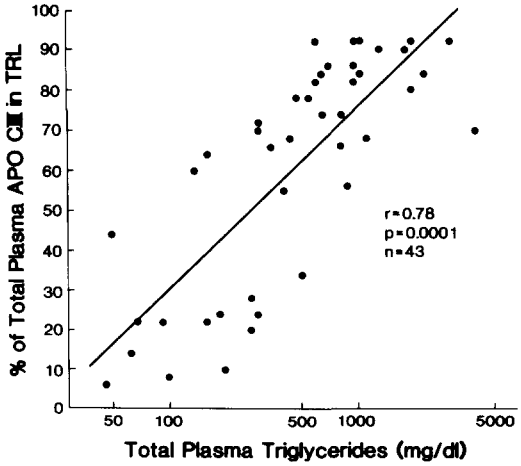

Fig. 5. The relationship between total plasma triglycerides and the percent of total plasma apoC-III in triglyceride-rich lipoproteins (TRL).

TABLE 1. ApoC-III as percent of HDL protein

Group	HDL_2	HDL_3	Total HDL
	% (mean ± SEM)		
Normal subjects (n = 15)	7.32 ± 1.23	1.61 ± 0.17	2.77 ± 0.35
Primary type IV HLP (n = 5)	2.38 ± 0.51[a]	0.60 ± 0.15[a]	0.98 ± 0.26[a]

[a] $P < 0.01$ (normal versus patients with primary type IV hyperlipoproteinemia (HLP)).

marizes the results of apoC-III in HDL. ApoC-III comprised 7.32 ± 1.23% of normal HDL_2 protein and was significantly higher ($P < 0.01$) than in HDL_2 isolated from subjects with a type IV lipoprotein phenotype (2.38 ± 0.51%). ApoC-III comprised 1.61 ± 0.17% of HDL_3 protein in normals versus 0.60 ± 0.15% in type IV individuals ($P < 0.05$). The percent of total HDL_2 + HDL_3 protein as apoC-III was 2.77 ± 0.35 in normals and 0.98 ± 0.26 in subjects with type IV hyperlipoproteinemia ($P < 0.01$). A significantly inverse correlation between total plasma TG and apoC-III as percent of HDL_2 protein ($r = -0.52$; $P = 0.019$) and HDL_3 protein ($r = -0.67$; $P = 0.0016$) was found. No systematic studies to assess the absolute concentrations of HDL apoC-III subspecies by IEF were conducted.

TRL apoC-III_0, C-III_1, and C-III_2 concentrations in plasma. The amount of apoC-III_0, C-III_1, and C-III_2 as percent of the total mass of apoC-III was calculated by planimetry of the scanned gels after the TRL apoC-III subspecies were separated by IEF of tetramethylurea-delipidated TRL. These ratios were then used to calculate the absolute concentration of apoC-III_0, C-III_1, and C-III_2 from the apoC-III concentration determined by RIA. These studies were done in 35 of the 43 subjects studied. **Table 2** shows the concentrations of TRL apoC-III subspecies in patients with three different lipoprotein phenotypes characterized by primary hypertriglyceridemia. TRL apoC-III_0, C-III_1, and C-III_2 levels were significantly ($P < 0.05$) higher in the three hypertriglyceridemic groups compared to controls. TRL apoC-III_0 levels in patients with types IV and V were comparable (2.4 ± 0.3 and 2.2 ± 0.6 mg/dl, respectively) although total plasma TG were significantly different (504 ± 55 versus 1646 ±249 mg/dl, respectively). However, TRL apoC-III_1 in patients with type V hyperlipoproteinemia (30.1 ± 4.6 mg/dl) was significantly higher ($P < 0.01$) than in patients with types IV or III hypertriglyceridemia (9.4 ± 1.4 and 7.8 ± 2.8 mg/dl, respectively). TRL apoC-III_2 levels were also significantly higher ($P < 0.01$) in plasma from type V patients (13.2 ± 2.1 mg/dl) than in type IV and III patients (6.3 ± 0.9 and 4.3 ± 1.6 mg/dl, respectively). **Fig. 6** shows the composite results of the relationship between total plasma TG and the absolute plasma concentrations of TRL apoC-III_0, C-III_1, and C-III_2. The vertical scale is identical for the three subspecies. A significant positive correlation was observed between total plasma TG and TRL apoC-III_0 ($r = 0.56$; $P = 0.0004$), apoC-III_1 ($r = 0.82$; $P = 0.0001$), and apoC-III_2 ($r = 0.76$; $P = 0.0001$). The apoC-III_1:TG regression line was the steepest. For a given TG interval, the change in apoC-III_1 was greater than that for apoC-III_0. The apoC-III_2:TG regression slope was also significantly steeper than that for apoC-III_0:TG. A statistical analysis of the regression lines showed that the slopes for apoC-III_1 and apoC-III_2 versus TG were significantly ($P < 0.01$) steeper than apoC-III_0 versus TG.

TABLE 2. Plasma TRL apoC-III subspecie concentrations in hypertriglyceridemic subjects

Group	TG	TRL C-III_0	TRL C-III_1	TRL C-III_2
		mg/dl[a]		
Normal (n = 5)	198 ± 8	0.3 ± 0.05	1.3 ± 0.5	0.6 ± 0.2
Type III (n = 3)	576 ± 113	0.7 ± 0.1	7.8 ± 2.8	4.3 ± 1.6
Type IV (n = 14)	504 ± 55	2.4 ± 0.3	9.4 ± 1.4	6.3 ± 0.9
Type V (n = 13)	1646 ± 249	2.2 ± 0.6	30.1 ± 4.6	13.2 ± 2.1

[a] Mean ± SEM.

DISCUSSION

A specific, accurate, sensitive and precise radioimmunoassay for quantitating total apoC-III in human plasma and its lipoprotein fractions has been developed. Analytical isoelectric focusing has been used to determine the individual proportions of the desialylated apoC-III (apoC-III_0) and each sialylated apoC-III subspecies (apoC-III_1 and apoC-III_2) in TRL. With the results obtained by these two techniques, absolute concentrations of TRL apoC-III_0, C-III_1, and C-III_2 were obtained. This approach can also be taken for the absolute measurement of C-III apoproteins in other lipoprotein fractions. The validation of these methods and their utilization as tools for the assessment of apoC-III in human plasma and its subfractions from normal and hyperlipidemic patients is discussed below.

Radioimmunoassay of total apoC-III

The RIA is specific for apoC-III as judged by lack of significant cross-reactivity with apoA-I, A-II, B (as LDL protein, d 1.030–1.050 g/ml), C-I, C-II, and lipoprotein-free plasma. The rabbit anti-apoC-III_1 cross-reacted completely with apoC-III_2. Furthermore, no change in the immunoreactive levels of apoC-III were found before and after in vitro desialylation of TRL apoC-III_2 and apoC-III_1 using the desialylating enzyme neuraminidase. Thus, the assay measures all apoC-III subspecies. Serial dilutions of plasma and apoC-III-containing lipoproteins (TRL, IDL, HDL_2, and HDL_3) showed displacement curves that were parallel to the standard curve, indicating immunologic identity of apoC-III in each lipoprotein class. The method is sensitive and detects apoC-III in the nanogram range. The accuracy of the method was examined by different approaches.

A), ApoC-III was measured in whole plasma and in the d < 1.006 g/ml and d > 1.006 g/ml fractions after ultracentrifugation. This procedure separated TRL from HDL. There was close agreement between the apoC-III concentration in whole plasma and the sum of concentrations observed in each subfraction. Inasmuch as ultracentrifugation separated TRL from HDL and LDL, the results indicate that any differences in the nature of binding of apoC-III to these lipoproteins did not alter the availability of immunologic sites for displacement of tracer.

B), When apoC-III_1 was added to whole plasma, the concentration of immunoreactive apoC-III was increased by an amount that would be expected by the apoC-III_1 addition. Thus, any added apoC-III_1 that bound to plasma lipoproteins (e.g., TRL and HDL) or remained free in solution was detected by the apoC-III_1 antibody.

C), TRL isolated from plasma by ultracentrifugation was delipidated with tetramethylurea. The TRL apoC-III_1 concentration was measured by RIA. Delipidated TRL apoC-III's were separated by analytical IEF and quantitated by running a concurrent series of gels on which different amounts of apoC-III_1 were applied. The gels were scanned and a standard curve relating the mass of apoC-III_1 applied to its densitometric area was plotted. This standard curve was used for estimating the total concentration of apoC-III liberated from TRL after tetramethylurea delipidation. Determination of TRL apoC-III concentrations by RIA and IEF after delipidation gave similar values. These data thus support the conclusion that all the immunoreactive sites are available to the apoC-III_1 antibody for displacement of the tracer.

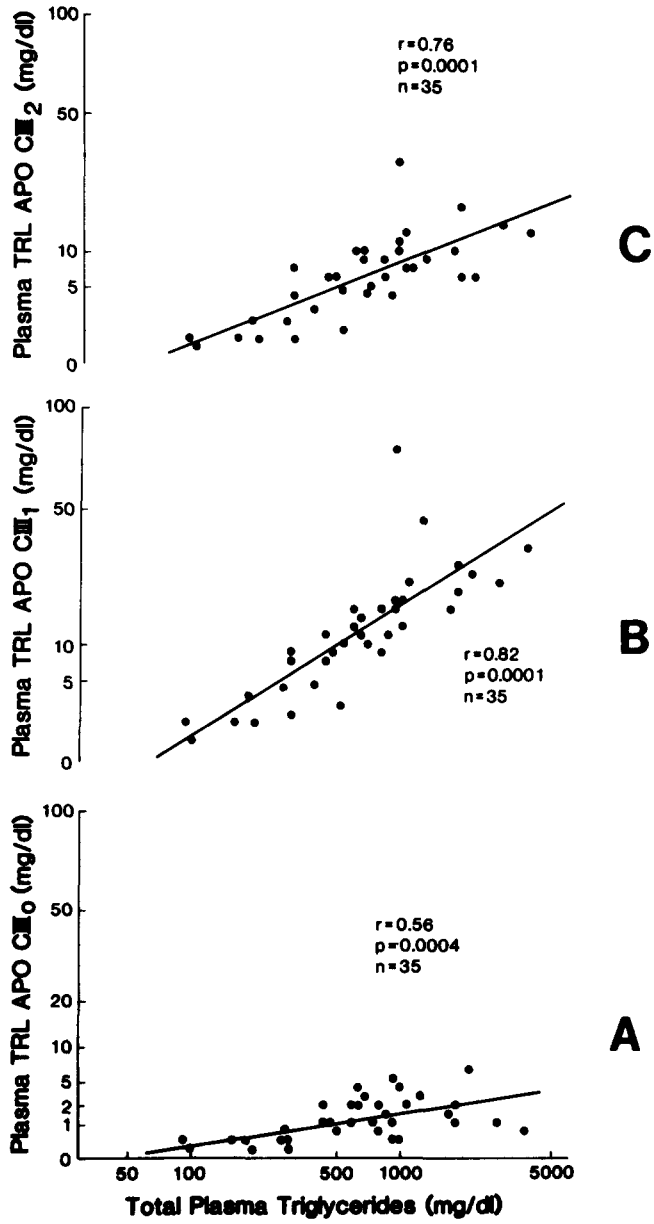

Fig. 6. Triglyceride-rich lipoproteins were isolated from plasma by preparative ultracentrifugation. Ratios of apoC-III_0, C-III_1, and C-III_2 to the total mass of apoC-III in TRL were obtained after scanning of gels obtained by analytical isoelectric focusing of delipidated TRL. ApoC-III was measured by radioimmunoassay. The data obtained by these two methods were used to compute absolute plasma levels of each TRL apoC-III subspecies. Panels A, B and C show the relationship between total plasma triglycerides and TRL apoC-III_0, C-III_1, and C-III_2 concentrations, respectively. Note the differences in slopes of the regression lines.

D), Because of reports that heating can increase the immunoreactive sites of apoA-I (25), we also conducted an experiment to determine the effect of heat on immunoassayable apoC-III. The results show that heating plasma at 52°C for 3 hr had no effect on apoC-III concentration in this assay.

E), The results of total plasma apoC-III concentration obtained by our RIA in normal and hypertriglyceridemic subjects are very similar to the results obtained by Schonfeld et al. (27) using a radioimmunoassay and those by Curry et al. (28) using the electroimmunoassay, and values obtained indirectly from the composition of VLDL and HDL proteins (29). Our results also indicate that lipoprotein apoC-III_1 antigenic sites are readily accessible to the antibody. Thus, the apoC-III_1 antibody used in this study appears to be similar in this regard to that raised by Mao et al. (30).

F), Adding further evidence to support the validity of the assay are the observed relationships between total plasma TG and *i*) total plasma apoC-III concentration (Fig. 3), *ii*) TRL apoC-III concentration (Fig. 4), *iii*) apoC-III distribution in TRL and TRL-free plasma (Fig. 5), and *iv*) the amount of HDL protein comprised by apoC-III. These relationships are the same as those determined by other types of in vitro and in vivo experimental evidence (3, 13, 27, 31–35).

The relationship between plasma triglycerides and the distribution of radioimmunoassayable apoC-III

Several in vivo and in vitro studies indicate that the important factors governing the distribution of certain apolipoproteins common to TRL and HDL are the mass ratio and the particle size of these lipoprotein classes. For example, the larger the ratio of TRL to HDL the greater is the proportion of total plasma apoC-II bound to TRL (31, 32). Increased total plasma TG concentrations are associated with an increase in number or size (or both) of TRL particles (36). Thus, with progressive hypertriglyceridemia, more apoC-III would be expected in TRL relative to HDL. In this study, total plasma TG correlated positively with total TRL apoC-III, and percent of total plasma apoC-III in TRL (Figs. 4 and 5). These results are consistent with the above-mentioned concept regarding the plasma distribution characteristics in the C apoproteins in general.

Age and sex did not influence apoC-III, its distribution in plasma, or its content in HDL or TRL. It is possible that if much larger populations are studied, differences may be found. In the present study, we have extended previous observations and assessed the relationship between total plasma TG and the amount of HDL_2 and HDL_3 protein comprised by apoC-III. The results indicate that normal HDL_2 protein was richer in apoC-III. Patients with hypertriglyceridemia (type IV lipoprotein phenotype) had subnormal amounts of apoC-III as a percent of HDL_2 and HDL_3 protein (Table 1). Additional data analysis revealed that total plasma TG bore an inverse relationship to apoC-III as percent of HDL_2 and HDL_3 protein. These observations support the concept that a progressive increase in TRL mass is associated with a progressive depletion of apoC-III in HDL. The reason for the observed higher content of apoC-III in HDL_2 than HDL_3 protein is not apparent in this study. One possibility is that the distribution of apoC-III in fasting plasma may be governed by the mass or size ratios of these two HDL subclasses such that the larger HDL_2 particles may have a greater affinity for apoC-III. Systematic studies are necessary to answer this question.

Analytical isoelectric focusing to determine relative ratios of apoC-III subspecies

A detailed description of this method and its validation has been published previously (19). In this communication, we utilized this analytical IEF method in combination with the RIA to obtain the absolute concentrations of each TRL apoC-III subspecies. This information cannot be obtained by sole use of either method. The information obtained by this approach is discussed below.

Total plasma triglycerides and TRL apoC-III_0, C-III_1 and C-III_2 concentrations

Using analytical IEF for measuring TRL apoC-III ratios, an abnormal preponderance of sialylated apoC-III_1 and C-III_2 relative to desialylated apoC-III_0 in TRL from type V hypertriglyceridemic patients was found (19). The new information obtained in this study was the data on absolute plasma concentrations of each TRL apoC-III subspecies. Total plasma TG correlated positively with total plasma TRL apoC-III (Fig. 4). However, the regression slopes for individual TRL apoC-III subspecies were different (Fig. 6). For a given plasma TG interval, the slopes for the sialylated apoC-III subspecies (apoC-III_1 and apoC-III_2) were significantly steeper than the desialylated TRL apoC-III_0. The slope for TRL apoC-III_1 versus plasma TG was the steepest (Panel B, Fig. 6). The data also indicated that the mean TRL apoC-III subspecie concentrations were significantly different in hypertriglyceridemics than in normal controls. The hypertriglyceridemic group was also heterogeneous. For example, TRL apoC-III_0 concentration was similar in the patients with types IV and V phenotypes (2.4 ± 0.3 versus 2.2 ± 0.6 mg/dl, respectively). Since progressive hypertriglyceridemia may be associated with alterations in TRL particle size, it is possible that the affinity of individual apoC-III's between chylomicrons, remnants, and VLDL may be different. The distribution behavior of apoC-III's

may be further influenced by the nature and amount of HDL or its subfractions. Another explanation may be that the synthesis or catabolism of individual apoC-III's may be regulated differently. These factors need to be examined carefully by a systematic investigation that was beyond the scope of the present study.

As alluded to earlier, apoC-III_1 and possibly other C apoproteins, oppose apoE-mediated hepatic clearance of TRL or their remnants (8–12). If these elegant experimental animals studies are relevant to human disease, the powerful correlation between total plasma TG and TRL apoC-III (especially apoC-III_1) observed in this study supports the possibility that TRL apoC-III levels may be related to the cause rather than the result of hypertriglyceridemia in man.

This research was supported by Public Health Service Grant HL-22618 and NIH NHLI-72-2914-L awarded by National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health and Human Services. The useful comments by Drs. Richard L. Jackson and Charles J. Glueck during the course of this investigation are appreciated. We thank the Core Laboratory of the Cincinnati Lipid Research Clinic for lipid quantitation and Ms. Wilma F. Roll for secretarial assistance.

Manuscript received 10 December 1980 and in revised form 9 March 1981.

REFERENCES

1. Jackson, R. L., J. D. Morrisett, and A. M. Gotto, Jr. 1976. Lipoprotein structure and metabolism. *Physiol. Rev.* **56:** 259–316.
2. Brewer, H. B., Jr., R. Schulman, P. Herbert, R. Ronan, and K. Wehrly. 1974. The complete amino acid sequence of alanine apolipoprotein (apo C-III), an apolipoprotein from human plasma very low density lipoproteins. *Biol. Chem.* **249:** 4975–4984.
3. Havel, R. J., J. P. Kane, and M. L. Kashyap. 1973. Interchange of apolipoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in man. *J. Clin. Invest.* **52:** 32–38.
4. Havel, R. J., V. G. Shore, and D. M. Bier. 1970. Role of specific glycopeptides of human serum lipoproteins in the activation of lipoprotein lipase. *Circ. Res.* **27:** 595–600.
5. La Rosa, J. C., R. I. Levy, P. N. Herbert, S. E. Lox, and D. S. Fredrickson. 1970. A specific apoprotein activator for lipoprotein lipase. *Biochem. Biophys. Res. Commun.* **41:** 57–62.
6. Havel, R. J., C. J. Fielding, T. Olivecrona, V. G. Shore, P. E. Fielding, and T. Egelrud. 1973. Co-factor activity of protein components of human very low density lipoproteins in the hydrolysis of triglycerides by lipoprotein lipase from different sources. *Biochemistry.* **12:** 1828–1833.
7. Brown, W. V., and M. L. Baginsky. 1972. Inhibition of lipoprotein lipase by an apoprotein of human very low density lipoprotein. *Biochem. Biophys. Res. Commun.* **46:** 375–381.
8. Shelbourne, F., J. Hanks, W. Meyers, and S. Quarfordt. 1980. Effect of apoproteins on hepatic uptake of triglyceride emulsions in the rat. *J. Clin. Invest.* **65:** 652–658.
9. Havel, R. J., Y. Chao, E. E. Windler, L. Kotite, and L. S. S. Guo. 1980. Isoprotein specificity in the hepatic uptake of apolipoprotein E and the pathogenesis of familial dysbetalipoproteinemia. *Proc. Natl. Acad. Sci. USA.* **77:** 4349–4353.
10. Windler, E., Y. Chao, and R. J. Havel. 1980. Regulation of the hepatic uptake of triglyceride rich lipoproteins in the rat. Opposing effects of homologous apolipoprotein E and individual C apolipoproteins. *J. Biol. Chem.* **255:** 8303–8307.
11. Windler, E., Y. Chao, and R. J. Havel. 1980. Determinants of hepatic uptake of triglyceride rich lipoproteins and their remnants in the rat. 1980. *J. Biol. Chem.* **255:** 5475–5480.
12. Sherrill, B. C., T. L. Innerarity, and R. W Mahley. 1980. Rapid hepatic clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor. *J. Biol. Chem.* **255:** 1804–1807.
13. Kashyap, M. L., L. S. Srivastava, C. Y. Chen, G. Perisutti, M. Campbell, R. F. Lutmer, and C. J. Glueck. 1977. Radioimmunoassay of human apolipoprotein C-II. A study in normal and hypertriglyceridemic subjects. *J. Clin. Invest.* **60:** 171–180.
14. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
15. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244:** 5687–5694.
16. Kane, J. P., T. Sata, R. L. Hamilton, and R. J. Havel. 1975. Apolipoprotein composition of very low density lipoproteins of human serum. *J. Clin. Invest.* **56:** 1622–1634.
17. Laemmli, U. 1970. Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London).* **227:** 680–685.
18. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89:** 114–123.
19. Kashyap, M. L., B. A. Hynd, K. Robinson, and P. S. Gartside. 1981. Abnormal preponderance of sialylated apo C-III in triglyceride rich lipoproteins in type V hyperlipoproteinemia. *Metabolism.* **30:** 111–118.
20. Lipid Research Clinics Program. National Institutes of Health, Education and Welfare. 1974. Publication No. (NIH) 75-628:9–50.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
22. Kashyap, M. L., B. A. Hynd, and K. Robinson. 1980. A rapid and simple method for measurement of total protein in the very low density lipoproteins by the Lowry assay. *J. Lipid Res.* **21:** 491–495.
23. Kane, J. P. 1973. A rapid electrophoretic technique

for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
24. Morell, A. G., C. J. A. Van den Hamer, I. H. Scheinberg, and G. Ashwell. 1966. Physical and chemical studies on ceruloplasmin. *J. Biol. Chem.* **241:** 3745–3749.
25. Karlin, J. B., D. J. Juhn, J. I. Starr, A. M. Scanu, and A. H. Rubenstein. 1976. Measurement of human high density lipoprotein apolipoprotein A-I in serum by radioimmunoassay. *J. Lipid Res.* **17:** 30–37.
26. Snedecor, G. R., and W. G. Cochran. 1967. *In* Statistical Methods. Iowa State University Press, Ames. 6th edition. 135–198.
27. Schonfeld, G., P. K. George, J. Miller, P. Reilly, and J. Witzum. 1979. Apolipoprotein C-II and C-III levels in hyperlipoproteinemia. *Metabolism.* **28:** 1001–1010.
28. Curry, M. D., W. J. McConathy, J. D. Fesmire, and P. Alaupovic. 1980. Quantitative determination of human apolipoprotein C-III by electroimmunoassay. *Biochim. Biophys. Acta.* **617:** 503–513.
29. Havel, R. J., J. L. Goldstein, and M. S. Brown. 1980. *In* Metabolic Control and Disease. P. K. Bondy and L. E. Rosenberg, editors. Saunders, Philadelphia. 8th edition. 399.
30. Mao, S. J. T., P. K. Bhatnagar, A. M. Gotto, Jr., and J. T. Sparrow. 1980. Immunochemistry of human very low density lipoproteins: apolipoprotein C-III. *Biochemistry.* **19:** 315–320.
31. Kashyap, M. L., L. S. Srivastava, R. C. Tsang, M. R. Taskinen, B. A. Hynd, G. Perisutti, D. W. Brady, C. J. Glueck, C. A. Ahumada, J. A. McCarthy, R. A. Sosa, and T. O. Reeds. 1980. Apolipoprotein C-II in type I hyperlipoproteinemia. A study in three cases. *J. Lab. Clin. Med.* **95:** 180–187.
32. Kashyap, M. L., L. S. Srivastava, B. A. Hynd, P. Gartside, and C. J. Glueck. 1979. The role of high density lipoprotein CII in triglyceride metabolism. *In* HDL Symposium. F. T. Lindgren, A. V. Nichols, and R. N. Krauss, editors. American Oil Chemists Society, Champaigne, IL. 42–51.
33. Schaefer, E. J., S. Eisenberg, and R. I. Levy. 1978. Lipoprotein apoprotein metabolism. *J. Lipid Res.* **19:** 667–687.
34. Kostner, G. M., J. R. Patsch, S. Sailer, H. Braunsteiner, and A. Holasek. 1974. Polypeptide distribution of main lipoprotein density classes separated from human plasma by rate zonal ultracentrifugation. *Eur. J. Biochem.* **15:** 611–621.
35. Eisenberg, S., D. W. Bilheimer, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. II. Studies on the transfer of apolipoproteins between plasma lipoproteins. *Biochim. Biophys. Acta.* **280:** 94–104.
36. Ruderman, N. B., A. L. Jones, R. M. Krauss, and E. Shafrir. 1971. A biochemical and morphologic study of very low density lipoproteins in carbohydrate-induced hypertriglyceridemia. *J. Clin. Invest.* **50:** 1355–1368.