Direct measurement of apoprotein C-III specific activity in ^{125}I-labeled very low density lipoproteins using immunoaffinity chromatography

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Abstract We have developed a technique for isolating apoprotein C-III by immunoaffinity chromatography, allowing the measurement of its specific radioactivity in lipoprotein fractions from small plasma samples. IgG specific for apoC-III was purified from goat antisera and bound to Sepharose. One ml of this gel (5 mg of IgG) bound 80–90 μg of apoC-III. The specific activity of apoC-III was determined by application of delipidated very low density lipoproteins to 1-ml columns and analysis of the protein eluted at pH 2.5 for mass and radioactivity. The coefficient of variation for apoC-III specific activity determination from ^{125}I-labeled VLDL was 4.3%. Minimal contamination of the eluates by apoproteins B, E, and C-II was confirmed by radioimmunoassay (0.3–1.2%). Following the injection of autologous ^{125}I-labeled VLDL, specific activity decay curves for VLDL apoC-III were biexponential, with the clearance of apoC-III being slower in hypertriglyceridemic subjects. These affinity columns can be used repeatedly and yield reproducible results. This technique should be useful for simultaneous studies of the turnover of several apoproteins in the same individual following a single injection of labeled autologous lipoprotein.—**Bukberg, P. R., N-A. Le, H. N. Ginsberg, J. C. Gibson, L. C. Goldman, and W. V. Brown.** Direct measurement of apoprotein C-III specific activity in ^{125}I-labeled very low density lipoproteins using immunoaffinity chromatography. *J. Lipid Res.* 1983. **24:** 1251–1260.

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The C-apolipoproteins of human plasma, designated apoC-I, apoC-II, and apoC-III, are small polypeptides of molecular weight 6000–9000. ApoC-III, 79 amino acids in length, is found in three glycosylated isoforms containing either two moles of sialic acid (apoC-III$_2$), one mole (apoC-III$_1$), or none (apoC-III$_0$) (1, 2). In fasting plasma, the C-apoproteins are associated primarily with very low density lipoproteins (VLDL, d < 1.006 g/ml) and high density lipoproteins (HDL, d 1.063–1.21 g/ml), constituting 40–60% of the total protein in the former and 5–10% of that in the latter. In vitro studies have demonstrated rapid exchange of labeled C-apoproteins between VLDL and HDL, with the final distribution governed by the relative proportions of these lipoproteins in the plasma or incubation mixtures (3). In addition to this steady state equilibration process, a redistribution of apoC from VLDL to HDL has been demonstrated to occur during heparin-induced lipolysis in vivo, and following hydrolysis of VLDL with post-heparin plasma or bovine milk lipoprotein lipase in vitro (4–6). Conversely, apoC has been shown to transfer from HDL to nascent VLDL and chylomicrons following secretion of these triglyceride-rich particles into lymph or plasma (7).

Although apoC-II and apoC-III appear to transfer between VLDL and HDL at similar rates, their respective metabolic roles are quite distinct. ApoC-II is a specific activator of lipoprotein lipase (LPL), the rate-limiting enzyme in chylomicron and VLDL-triglyceride hydrolysis, and absence of this apoprotein has been reported to result in severe hypertriglyceridemia (8, 9). The exact metabolic role of apoC-III, however, remains less clear. The observation that apoC-III appears to inhibit apoC-II-induced activation of LPL in vitro (10) has prompted speculation that the elevated ratios of apoC-III relative to apoC-II found in patients with hypertriglyceridemia may contribute to faulty VLDL clearance (11). Subsequent studies by Eisenberg et al. (6), however, demonstrated that the ratio of apoC-III to apoC-II in VLDL was unaffected by the extent of lipolysis in an in vitro system using bovine milk LPL.

Abbreviations: apoC-III, apoprotein C-III; VLDL, very low density lipoproteins; HDL, high density lipoproteins; LPL lipoprotein lipase; IgG, immunoglobulin G; TG, triglyceride; FCR, fractional clearance rate; SDS, sodium dodecyl sulfate; EDTA, disodium ethylenediamine tetraacetate; RIA, radioimmunoassay; SA, specific radioactivity.

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More recent investigations into the physiologic role of apoC-III have focused not on its possible modulation of LPL activity, but rather on its effects on the interaction of VLDL and remnant particles with cell membranes. Studies by Shelburne et al. (12) have demonstrated that incubation of rat lymph chylomicrons and triglyceride emulsions with excess human apoC-III exerts a pronounced inhibitory effect on the apoE-mediated hepatic clearance of these particles in a nonrecycling rat liver perfusion system. Similar findings were reported by Windler, Chao, and Havel using rat apoC-III (13), although in this latter study the inhibitory effect was not limited to apoC-III, but occurred with apoC-II as well.

Because of their similar molecular weights and solubility characteristics, purification of the individual C-apoproteins from the limited amounts of material available in a small plasma sample is extremely difficult. This has discouraged previous attempts to examine the turnover of apoC-III in human subjects. The study of Berman et al. (14) provided data on the turnover of the C-apolipoproteins as a group, but failed to fractionate the individual proteins. A method for performing turnover studies of apoC that utilizes isoelectric focusing to separate apoC-II from C-III, however, has recently been described (15). This is nonetheless a cumbersome technique, and its precision is limited by the total amount of protein that can be analyzed on each gel. We now present a new method for studying the metabolism of apoC-III that employs immunoaffinity chromatography to isolate this apoprotein from lipoprotein fractions in small plasma samples for determination of specific radioactivity. A radioimmunoassay for quantitation of the apoprotein is also described.

METHODS

Materials

Sepharose CL-4B, DEAE-Sephacel, Sephadex G-100 and G-150 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Bovine albumin (Fraction V), ovalbumin, lactoperoxidase, Tween-20, and Triton X-100 were purchased from Sigma Chemical Company (St. Louis, MO). Cyanogen bromide was obtained from Aldrich Chemicals (Milwaukee, WI), polyethylene glycol-6000 from J. T. Baker Chemical Co. (Phillipsburg, NJ), sodium decyl sulfate from Eastman Kodak Co. (Rochester, NY), and aprotinin (Trasylol^{R}) from Mobay Chemicals (New York, NY). Carrier-free ^{125}I was purchased from ICN Chemicals and Radioisotopes (Irvine, CA). All other reagents were routinely obtained from Fisher Scientific Company (Fair Lawn, NJ).

Purification of apoC-III

VLDL was isolated from plasma of human donors with Type IV hyperlipoproteinemia by ultracentrifugation at density 1.006 g/ml and washed once by reflotation at this density. The preparations were lyophilized, and then delipidated with heptane and ethanol–diethyl ether as previously described (16). The pellet remaining after delipidation was dissolved in freshly deionized 6 M urea containing 0.01 M Tris-HCl and 5 mM sodium decyl sulfate (pH 8.2), and chromatographed over a 2.5 × 100 cm column of Sephadex G-150. The lower molecular weight fractions containing the C-apoproteins were pooled, dialyzed against NH_4HCO_3, and lyophilized. The freeze-dried material was then redissolved in 6 M urea, 0.01 M Tris-HCl (pH 9.5), and fractionated on DEAE-Sephacel (17) using a linear gradient developed between buffers of 6 M urea, 0.01 M Tris-HCl (pH 8.2) and 6 M urea, 0.1 M Tris-HCl, 0.03 M NaCl (pH 8.2).

Antisera

Of the apoC-III isoforms isolated by ion exchange chromatography, only apoC-III$_2$ routinely yielded a single band on analytical isoelectric focusing (18), and was therefore utilized as immunizing antigen. Antisera were raised in both rabbits and goats, using a loading dose of 300 μg of apoC-III$_2$ emulsified in an equal volume of complete Freund's adjuvant. Animals showing a detectable antibody titer were boosted every 6–8 weeks with 150 μg of apoC-III$_2$ emulsified with incomplete Freund's adjuvant and bled 7–10 days later. Rabbit antisera were used for radioimmunoassays. Goat antisera were found more practical for preparation of the anti-apoC-III affinity columns because of the large quantities of IgG required.

Radioimmunoassay of apoC-III

ApoC-III$_2$ was utilized as both standard and tracer for the assay. Radioiodination was performed using lactoperoxidase (19), yielding an average specific activity of 25–35 μCi/μg. Immediately following iodination, the bulk of unreacted ^{125}I was removed by dialysis of the tracer against 0.15 M NaCl, 0.1 mg/ml EDTA (pH 7.4) for 60–90 min. The preparation was then dialyzed overnight against 6 M urea, 0.01 M Tris-HCl (pH 8.2) containing 0.1% ovalbumin and 5 mM sodium decyl sulfate, and chromatographed over a 1.5 × 90 cm column of Sephadex G-100 equilibrated in the same urea buffer. (Portions of the tracer preparation could also be frozen at −70°C for gel filtration at a later date.) Fractions containing the major peak of radioactivity from the G-100 column were pooled and dialyzed against 0.1 M borate, 5 mM sodium decyl sulfate (pH 8.5), and aliquots

were frozen at −70°C until used in the radioimmunoassay. Such aliquots were generally stable for 2–3 weeks and could be used directly in the RIA after thawing without the need for rechromatography.

Assays were carried out in 0.1 M borate buffer (pH 8.5) containing 0.1% ovalbumin, 0.01% Triton X-100, and 50 KIU/ml aprotinin (Trasylol^{R}). Glass tubes (12 × 75 mm) were rinsed in a solution of 0.1% Tween-20 and dried before use. To each tube were added the following: 100 μl of ^{125}I-labeled apoC-III (approximately 15,000 cpm), 100 μl of rabbit anti-apoC-III serum (final dilution 1:2500–1:4000), 100 μl of nonimmune rabbit serum diluted 1:400, and 100 μl of the sample (standard or unknown) appropriately diluted in assay buffer. Tubes were incubated initially at 37°C for 2.5 hr and then at 4°C for 40 hr, after which 200 μl of goat anti-rabbit antiserum (diluted 1:10) was added. After an additional incubation period (20 hr) at 4°C, 600 μl of a 20% solution of polyethylene glycol (PEG) in 0.1 M borate (pH 8.5) was added (20) and the tubes were centrifuged at 2500 rpm for 30 min at 4°C in a Beckman J6-B centrifuge (Beckman Instruments, Palo Alto, CA). The supernates from each tube were decanted, and the precipitates were washed by addition of 0.5 ml of a 10% solution of PEG; this was followed by centrifugation and decantation as before. Both standards and unknowns were assayed in duplicate. Tubes not containing anti-apoC-III antiserum were included in each assay to quantitate nonspecific binding (approximately 5%) and these counts were subtracted from total counts for each tube to determine net counts.

Standard curves were linearized using logit-log transformation of B/B_0 values in which B = net counts in tube containing standard or unknown, B_0 = net counts in absence of standard, and logit $B/B_0 = \log((B/B_0)/(1 - B/B_0))$ (21). Standard curves were plotted with logit B/B_0 on the ordinate and log (apoC-III concentration in ng/ml) on the abscissa, and the slope and intercept of the regression line were determined.

Preparation of anti-apoC-III affinity columns

Purification of specific IgG. IgG specific for apoC-III was isolated by adsorption of goat antiserum with apoC-III-Sepharose. The latter was prepared by covalently binding apoC-III$_1$ to cyanogen bromide-activated Sepharose CL-4B (50 mg CNBr/ml of gel) at a concentration of 1 mg apoprotein per ml of gel. Coupling was carried out at 4°C for 16 hr in a buffer of 0.5 M NaCl, 0.1 M $NaHCO_3$ (pH 8.3) containing 5 mM sodium decyl sulfate (22, 23); the coupling efficiency was over 95%. After washing the gel with several volumes of coupling buffer, 1 M glycine (pH 8.6) was added to bind unreacted sites. Nonspecifically bound protein was removed by alternating washes of 10 gel vol of coupling buffer with equal amounts of 0.5 M NaCl, 0.1 M sodium acetate at pH 4.0 (three cycles). The gel was then equilibrated with 0.1 M NaCl, 50 mM PO_4, pH 7.4 (PBS) containing 0.02% sodium azide for storage at 4°C. Prior to its initial use, the gel was incubated for 1 hr with nonimmune goat serum (diluted 1:10) to saturate nonspecific binding sites. The gel was then exhaustively washed with PBS, followed by 10–20 gel vol of 3 M sodium thiocyanate, and re-equilibrated in PBS.

For isolation of specific IgG, aliquots of apoC-III-Sepharose (2 ml) were each combined with 12–15 ml of goat antiserum and placed in 50-ml plastic screw-capped tubes. The suspensions were diluted with PBS to a total volume of 30 ml, and the tubes were incubated for 3 hr at 4°C on a mechanical shaker (Eberbach, Ann Arbor, MI). The contents of all tubes were then decanted into a single 1.5 × 10 cm plastic "Econo-column" (Bio-Rad, Richmond, CA). The gel was first washed extensively with PBS, and then with a higher ionic strength buffer (0.5 M NaCl, 25 mM PO_4, pH 7.4) to remove nonspecifically bound protein. Specific IgG was then eluted with 0.2 M glycine at pH 2.5, immediately dialyzed against PBS to minimize denaturation, and stored at −70°C until further use.

Preparation of anti-apoC-III affinity column. Specific IgG isolated as above was then covalently bound to cyanogen bromide-activated Sepharose CL-4B (60 mg CNBr/ml of gel) at a concentration of 5.5 mg of IgG per ml of gel. The coupling reaction was carried out for 16 hr at 4°C in a buffer of slightly acid pH (0.5 M NaCl, 25 mM PO_4, pH 6.5) to minimize the potential for multi-point attachment of the IgG (23). As before, 1 M glycine (pH 8.6) was utilized to bind unreacted sites, and nonspecifically bound protein was removed by washing sequentially with 0.5 M NaCl, 0.1 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl, 0.1 M sodium acetate (pH 4.0). The anti-apoC-III-Sepharose was then equilibrated with 0.1 M NaCl, 0.1 M borate at pH 8.0 (borate buffer). Nonspecific binding sites were saturated prior to initial use of the gel by application of a 0.1% solution of ovalbumin in this buffer. After washing the gel free of residual ovalbumin, 10 gel vol of 0.2 M glycine (pH 2.5) were applied, followed by equal volumes of 3 M sodium thiocyanate. The gel was then re-equilibrated with borate buffer. Several such elution cycles (three to five) were carried out until leaching of IgG or ovalbumin was no longer detectable on analysis of the eluate by the method of Lowry et al. (24).

Affinity chromatography of apoC-III. Aliquots of VLDL containing 250–400 μg of total protein were delipidated prior to application to the immunoaffinity columns. Lipoproteins were first treated with 20–30 vol of acetone, vortexed vigorously, sonicated for 1 min in a water bath

(Bransonic-52, Branson, Shelton, CT), and centrifuged at 2500 rpm for 30 min at 4°C. The acetone was decanted, and the identical procedure was repeated twice with 20 vol of isopropanol. After decanting the second wash of isopropanol, the pellet was resuspended in 1 ml of borate buffer containing 0.02% Triton X-100. (This detergent was chosen in order to promote the resolubilization of apoC-III while simultaneously limiting that of apoB; we were particularly concerned about nonspecific adsorption of apoB to column surfaces during subsequent affinity chromatography.) A brief period of sonication in a water bath was usually necessary to break up the delipidated pellet, and the tubes were then allowed to incubate overnight at 4°C. The next day, the suspensions were transferred to 1.5-ml microfuge tubes and centrifuged at 10,000 rpm for 20 min (Microfuge-B, Beckman Instruments) to remove visible precipitate and microaggregates.

The supernates were diluted to 5 ml total volume with additional borate buffer containing 0.02% Triton and transferred to disposable plastic Econo-columns (Bio-Rad) containing 1 ml of anti-apoC-III-Sepharose (binding capacity 80–90 μg of apoC-III). The apoprotein mixture was allowed to run slowly through the gel over a period of 60–90 min. Each column was then washed with 10–15 ml of the same borate/Triton buffer, followed by 20–30 ml of a buffer containing 0.5 M NaCl, 0.1 M borate, 0.01% Triton (pH 8.0) to remove nonspecifically bound protein. ApoC-III was eluted by addition of 3.0 ml of 0.2 M glycine (pH 2.5) containing 0.01% Triton; after discarding the initial 0.5 ml, the subsequent 2.5 ml of effluent containing the apoC-III was collected. For determination of specific radioactivity (SA), measured aliquots of the apoC-III eluate were counted directly in a Packard autogamma spectrometer (Packard Instruments, Downers Grove, IL); samples were counted for 10,000 total counts. Protein mass was determined on aliquots of the same eluate either by a modification of the Lowry procedure (25) or by radioimmunoassay. For the modified Lowry method, bovine albumin in 0.2 M glycine, 0.01% Triton (pH 2.5) was utilized as standard.

The entire chromatographic procedure was carried out at 4°C. After elution of the apoC-III, columns were washed with an additional 10 ml of 0.2 M glycine and with 20 ml of 3 M sodium thiocyanate before being re-equilibrated and stored in borate buffer containing 0.02% sodium azide.

Turnover studies

The method was tested by performing VLDL apoC-III turnover studies in four male subjects, two with normal lipoprotein levels and two with Type IV hyperlipoproteinemia. One normal subject underwent repeated studies 4 months apart under identical conditions. Clinical data on the four subjects is given in **Table 1.** Informed consent was obtained from each subject prior to the study.

The protocol used for performing the turnover studies has been previously described in detail (26). Briefly, subjects were admitted to the Mount Sinai Hospital General Clinical Research Center, and placed on a weight-maintaining diet consisting of 45% carbohydrate, 40% fat, and 15% protein with a polyunsaturated/saturated fat ratio of 0.4 and a cholesterol content of 150 mg per 1000 kcal. After a 4–7-day stabilization period, they were fasted overnight and underwent plasmapheresis of one unit of blood (1.0 mg/ml EDTA) the following morning. VLDL was isolated by ultracentrifugation at density 1.006 g/ml, washed during a second ultracentrifugation (26), and iodinated with ^{125}I or ^{131}I by a modification (27) of the iodine monochloride method (28). All procedures were performed using strict aseptic techniques.

Each subject received a saturated solution of potassium iodide, three drops three times daily, starting 1 day prior to injection of radiolabeled VLDL and continuing for 3 weeks. Eight hours prior to injection, subjects were switched to a fat-free liquid formula diet (75% carbohydrate, 25% protein, 60% of maintenance calories) which was given in equal fractions every 3 hr for the duration of the turnover study (29). Following the injection of 50 μCi of ^{125}I-labeled VLDL, 17 timed blood samples (10 ml each) were obtained (with EDTA, 1.5 mg/ml) over the next 72 hr (5 min, 30 min, 1, 1.5, 2, 4, 6, 9, 12, 15, 18, 24, 30, 39, 48, 60, and 72 hr). VLDL were isolated from 3 ml of plasma taken at each of these time points by ultracentrifugation at density 1.006 g/ml using a 40.3 rotor at 39,000 rpm for 16–20 hr at 10°C.

Plasma cholesterol and triglyceride concentrations were measured by specific enzymatic methods using an ABA-100 Analyzer (Abbott Laboratories, Chicago, IL). Lipoprotein cholesterol levels were determined according to Lipid Research Clinic methodology (30), except that dextran sulfate and $MgCl_2$ were used to precipitate the lipoproteins of density less than 1.063 g/ml before measurement of HDL-cholesterol (31).

TABLE 1. Clinical data on experimental subjects

Subject	Age	% Ideal Body Weight	Plasma TG	Total Chol	HDL Chol
				mg/dl	
1	53	106	49	174	34
2	29	120	46	126	31
3	53	119	487	231	22
4	57	135	360	200	25

Data analysis

For each patient, the specific activity values for apoC-III at each time point were normalized with respect to the specific activity of the 5-min sample. The characteristics of the decay curves were then determined by the curve-peeling method of Matthews (32) in terms of a sum of two exponentials of the form $y(t) = A_1e^{-b_1t} + A_2e^{-b_2t}$. In this context, the terms b_1 and b_2 would correspond to the slopes of the two log-linear components of the decay curve. A_1 and A_2 may then be interpreted as the percent of the sample pool having an apparent half life of $0.693/b_1$ and $0.693/b_2$, respectively, and $A_1 + A_2 = 100\%$.

Assuming that the tracer was injected into a homogeneous pool, an estimate of the turnover time for apoC-III could be derived from the sum of exponentials (33). A more commonly used kinetic parameter, the fractional clearance rate (FCR_p), is the inverse of the turnover time and represents the fraction of the plasma pool irreversibly cleared per unit time. Thus, for a decay curve described by a sum of two exponential terms in the form given above, we have (32, 33):

$$FCR_p = 1/(\text{plasma turnover time})$$

$$= (A_1 + A_2)/(A_1/b_1 + A_2/b_2).$$

Since only the plasma pool is being sampled, it should be emphasized that this estimate for FCR_p assumes that irreversible degradation is occurring solely from the plasma space. For a more complex system, this estimate could be very different from the true FCR of VLDL apoC-III.

RESULTS

Radioimmunoassay for apoC-III

Maximum binding for the ^{125}I-apoC-III_2 tracer generally ranged from 85–90%, with nonspecific binding approximately 5%. A typical displacement curve using apoC-III_2 as standard is shown in **Fig. 1.** The working range for the standard curve was 1–100 ng/ml. Displacement of the apoC-III_2 tracer by apoproteins C-II, B, E, A-I, and human albumin were each less than 1% of that observed with an equivalent mass of apoC-III. Logit-log transformations of displacement curves obtained for dilutions of VLDL, HDL, and whole plasma are also shown in Fig. 1. The computer-derived slopes for these curves were similar to that of the apoC-III_2 standard. When displacement curves obtained using apoC-III_1 and apoC-III_2 were compared, the slopes and intercepts derived from logit-log transformation of each curve were not significantly different. A similar comparison was not performed for apoC-III_0 because this

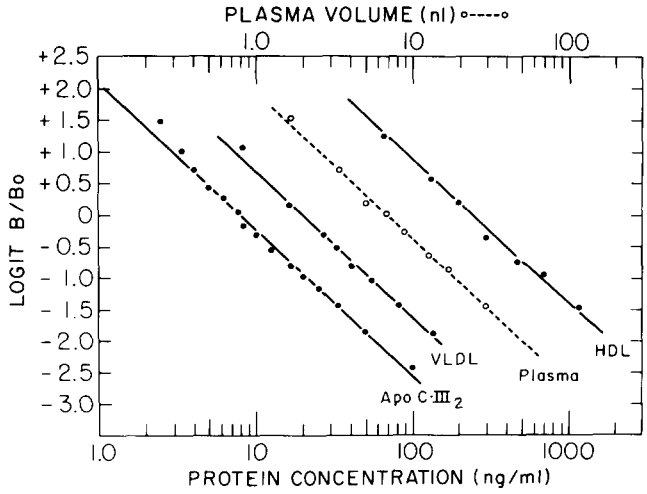

Fig. 1. Displacement of ^{125}I-labeled apoC-III and by serial dilutions of human VLDL, HDL, and whole plasma under conditions used for apoC-III radioimmunoassay.

is a minor isoform of apoC-III and is extremely difficult to obtain in pure form by standard chromatographic techniques. Nonetheless, the demonstration of parallelism between the displacement curves for apoC-III_2 and that for whole VLDL (containing a mixture of apoC-III isoforms) suggests that apoC-III_0 must have behaved identically to the other apoC-III isoforms following serial dilution.

The inter-assay coefficient of variation, assessed by running six quality control sera in at least 13 assays over a 6-month period, averaged 9.3 ± 1.2% (mean ± SD). Intra-assay variation, determined by running six replicates of eight different sera in the same assay, averaged 4.0 ± 1.4%.

To evaluate the effect of delipidation, plasma samples were obtained from seven subjects with total triglyceride levels ranging from 32 to 490 mg/dl, and apoC-III concentration was measured in each sample before and after delipidation (of whole plasma) with acetone and isopropanol. For each plasma considered separately, ratios of apoC-III concentrations in the delipidated to that in the nondelipidated sample averaged 0.95 ± 0.05. This agrees with the 5.1% estimated loss of apoC-III during the delipidation procedure itself, as measured by the recovery of a tracer amount of ^{125}I-labeled apoC-III added to aliquots of four plasma samples prior to delipidation.

Levels of apoC-III were measured in a series of plasma dilutions to which known quantities of the apoC-III_2 standard were added. The ratio of the observed to expected concentration of apoC-III for the ten samples, which covered a range of C-III values from 3.2 to 32 ng/ml, averaged 1.14 ± 0.09.

In a second series of recovery experiments, aliquots

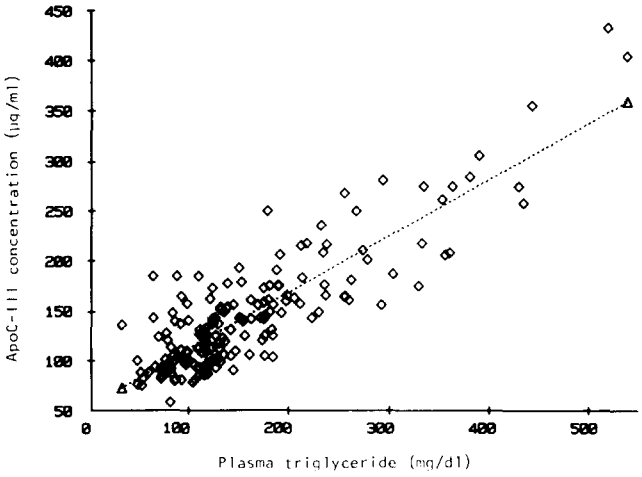

Fig. 2. Relationship between total plasma triglyceride and apoC-III concentration as determined by radioimmunoassay in sera from a group of 176 middle-aged adult males ($r = 0.863$, $P < 0.001$).

of three different plasma samples were subjected to sequential ultracentrifugation at densities 1.006, 1.019, 1.063, and 1.21 g/ml. The ratios of the summed apoC-III concentrations in the VLDL, IDL, LDL, HDL, and d > 1.21 g/ml bottom fractions to the apoC-III level measured in the original plasma were 1.11, 0.96, and 0.95. Less than 2.5% of the total plasma apoC-III was found in the d 1.21 g/ml fraction after the sequential ultracentrifugation.

Levels of apoC-III were measured in serum samples from a group of 176 adult male volunteers[2] (mean age 55, SD = 12.7 years). Levels of total plasma triglyceride in this population averaged 158 ± 91 mg/dl (range 32–538). The apoC-III concentration averaged 144.8 ± 60.2 µg/ml (range 59–435) for the group as whole, and **Fig. 2** shows the correlation of apoC-III concentration with plasma TG level ($r = 0.863$, $P < 0.001$). For the subset of 141 normotriglyceridemic subjects (plasma TG < 200 mg/dl), the mean level of apoC-III was 123.1 ± 32.9 µg/ml. This mean value is similar to those previously reported by Schonfeld et al. (34) (150 µg/ml) and by Kashyap et al. (35) (110 µg/ml). Although our control population consisted only of male subjects, neither of the latter studies detected any difference in mean apoC-III levels between males and females.

Isolation of apoC-III by affinity chromatography

In the purification of IgG specific for apoC-III, the binding capacity of the apoC-III-Sepharose was approximately 3 mg of IgG/ml of gel. Purity of the IgG eluted from the gel was confirmed by SDS-polyacrylamide gel electrophoresis (36), and its activity against apoC-III was demonstrated by double immunodiffusion. Immunoprecipitation experiments using ^{125}I-labeled apoC-III showed the specific IgG to have a binding capacity of at least 22.5 µg of C-III/mg of IgG.

Preparation of the anti-apoC-III-Sepharose. The efficiency of the coupling reaction in which the specific IgG was bound to Sepharose was approximately 90%, yielding a final concentration of 5 mg of IgG per ml of gel. The capacity of the anti-apoC-III-Sepharose, determined by application of a saturating quantity of apoC-III, extensive washing of the column, and measurement of the apoprotein eluted, was 80–90 µg of apoC-III/ml of gel.

Validation of the anti-apoC-III affinity columns. Although contamination of the eluates by small amounts of IgG and ovalbumin were evident during the initial use of the columns, leaching of these proteins ceased to be a problem after several elution cycles, as confirmed by analysis of the eluates on SDS-polyacrylamide gels (36). The eluate was analyzed by isoelectric focusing, and samples of these gels are shown in **Fig. 3.** Bands corresponding to each of the three isoforms of apoC-III are visible in the column eluate; these bands were identified by characteristic pI values and comparison with apoC-III standards purified by DEAE chromatography. ApoC-II was seen to be the dominant protein in the fraction that failed to bind to the column, but was absent from the eluate of the bound fraction. Subsequent analysis of eluate samples for the presence of apoC-II by specific radioimmunoassay indicated an average contamination of 1.2%. Contamination of the apoC-III eluates by apoproteins B and E, assessed by specific radioimmunoassays performed in our laboratory,[3] ranged from 0.3 to 0.8%.

ApoC-III eluted from the affinity columns remained sufficiently stable at pH 2.5 to allow determination of mass either by RIA or by the modified Lowry method (25). In fact, apoC-III concentrations in the eluate were often 10–15% higher when measured by the former technique. Simple exposure of the apoprotein to acidic pH cannot by itself have accounted for this difference since incubation of the apoC-III RIA standard for 24 hr in the pH 2.5 buffer did not increase its apparent immunoreactivity. More likely, the affinity chromatography procedure selected a population of apoC-III molecules that was completely immunoreactive; apoC-III purified in this fashion would then give higher readings

[2] These samples were obtained from a group of male volunteers at a steel manufacturing plant in West Virginia and were kindly provided by Dr. Marion Moses of the Department of Environmental Sciences.

[3] Gibson, J. C., A. Rubinstein, P. R. Bukberg, and W. V. Brown. Unpublished data.

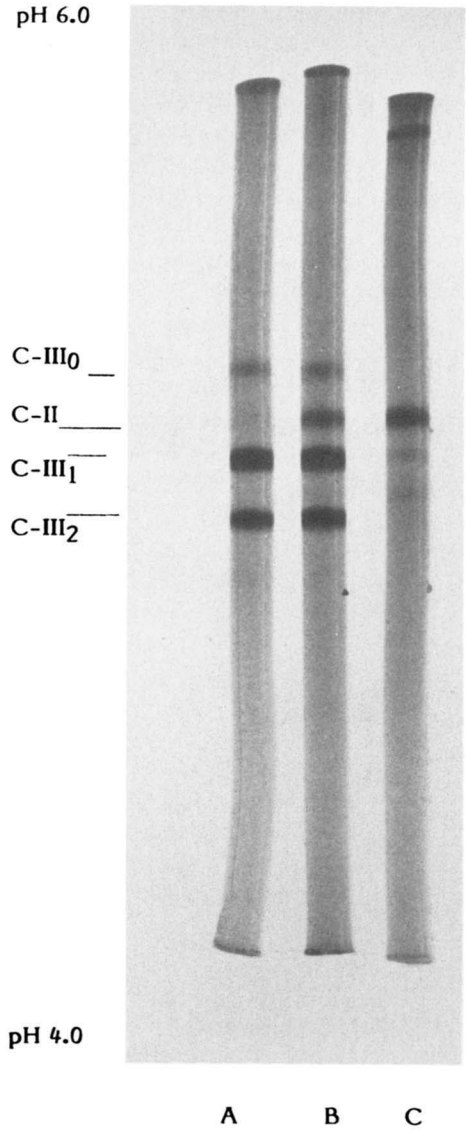

Fig. 3. Analytical isoelectric focusing of the apolipoproteins C from VLDL as applied to and eluted from anti-apoC-III affinity columns: (A) fraction bound to and eluted from affinity column, (B) all apolipoproteins C as isolated from VLDL by Sephadex G-150 chromatography (apoC-I is absent from this control gel since it does not focus in this pH range), and (C) material applied but not binding to column.

in the RIA relative to an apoC-III standard which may have suffered some loss in immunoreactivity during storage.

Reproducibility of the column method for measurement of the apoC-III specific activity was assessed by delipidation of six separate aliquots of ^{125}I-labeled VLDL at each of two protein loads. Delipidation and analysis of 400 μg of VLDL protein yielded 72.1 ± 6.4 μg apoC-III of specific activity 242.4 ± 13.5 cpm/μg; 250 μg of VLDL protein yielded 45.4 ± 4.9 μg apoC-III of SA 249.0 ± 7.8 cpm/μg.

Turnover studies

Normalized apoC-III specific activity decay curves from the four subjects are shown in **Fig. 4.** The apparent rate of clearance of apoC-III was significantly slower in the two hypertriglyceridemic subjects. Thus, 30–40% of the initial apoC-III radioactivity remained in the plasma VLDL of these individuals 60 hr after a bolus injection of ^{125}I-labeled VLDL, compared to the 10–15% remaining in the VLDL of normal control subjects at a similar point in time. When the curve-peeling method described by Matthews (32) was used to analyze the characteristics of the decay curves, two log-linear phases could be demonstrated in all subjects. It is of interest to note that while the half-lives of the second component were shorter for the normal subjects (23.4 and 27.6 hr) than for the hypertriglyceridemic individuals (39.5 and 51.3 hr), the half-lives of the first components were similar in the two groups (**Table 2**).

Assuming that the biphasic nature of the apoC-III decay curve resulted from an equilibration process between the plasma pool and extravascular space, and that irreversible loss of apoC-III occurred solely via the

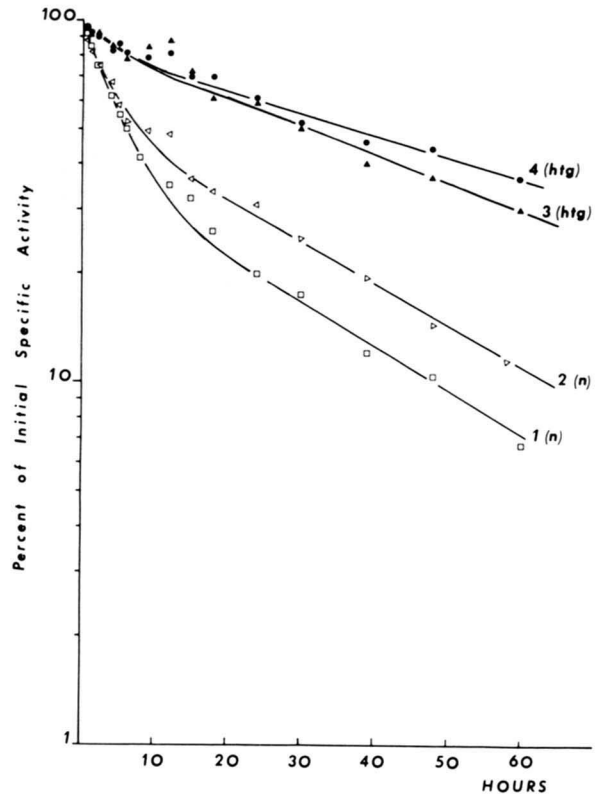

Fig. 4. Decay curves of VLDL apoC-III specific activity following the injection of ^{125}I-labeled VLDL in two normolipidemic (n) and two hypertriglyceridemic (htg) subjects. Data is expressed as percent of the specific activity present at 5 min after injection of the tracer (initial specific activity) with the 30-min sample as the first point shown.

TABLE 2. Exponential components of apoC-III specific activity decay curves

Subject	Fast Component[a]		Slow Component[a]		
	Percent[b]	Half-life[c]	Percent[b]	Half-life[c]	FCR
		hr		*hr*	hr^{-1}
1 Normal	60.87	2.85	39.13	23.42	0.058
2 Normal	47.09	2.52	52.91	27.61	0.040
3 HTG	21.43	3.30	78.57	39.50	0.022
4 HTG	18.13	2.88	81.87	51.34	0.016

[a] Fast and slow components correspond to the exponentials used to describe the decay curve as defined in the text.

[b] The percent of the fast and slow components refers to the parameters A_1 and A_2, respectively.

[c] Half-lives are defined as the ratio of 0.693 to the corresponding slopes b_1 and b_2.

plasma pool, we could estimate the overall fractional clearance rate (Table 1). The estimated FCR_p values for the hypertriglyceridemic subjects (0.016 and 0.022 hr^{-1}) were approximately half those obtained for the control individuals (0.040 and 0.058 hr^{-1}). Reproducibility of the turnover procedure was demonstrated in subject No. 2, in whom repeated studies under identical conditions 4 months apart yielded similar values for the FCR_p of apoC-III (0.040 and 0.045 hr^{-1}).

DISCUSSION

The isolation of apoC-III by affinity chromatography permits the accurate measurement of the specific activity of this apoprotein in lipoprotein fractions from small plasma samples. The affinity columns can be used repeatedly, and yield reproducible results over a wide range of protein masses. Apoprotein can be isolated in quantities ranging from 10–300 μg using less than 3 ml of gel. The small column size simplifies the task of running several columns at the same time, and allows one to collect the eluate in a very small volume. Limiting the quantity of gel also reduces the potential for nonspecific binding of contaminating apoproteins. Furthermore, the method is highly efficient in its use of material; unbound apoproteins should suffer no denaturation during passage through the columns, and can easily be recovered for subsequent isolation, as in other immunoaffinity procedures. The technique is therefore ideally suited to the simultaneous study of the turnover of several apoproteins in the same individual.

In the case of apoC-III, the initial isolation of IgG specific for the apoprotein of interest improved the binding capacity at least 10-fold over that of a whole IgG fraction prepared from the original antiserum by sodium sulfate precipitation. In addition, adsorption of antisera with apoprotein-Sepharose allows one to restrict the IgG population to those antibody molecules of an affinity appropriate for the elution scheme. Molecules of IgG with lower affinity are removed by extensively washing the antiserum-gel complex with high ionic strength salt solution prior to elution of the desired IgG at pH 2.5. The quantities of antiserum required for this procedure are best obtained by immunizing large animals such as goats or sheep.

Previous studies of apoC metabolism have utilized a variety of gel electrophoretic techniques to isolate these apoproteins for determination of specific activity. Reliable separation of apoC-II from the isoforms of apoC-III, however, has only been accomplished by isoelectric focusing (15). This technique is cumbersome to perform, requiring precise localization and slicing of each band from the gel prior to gamma counting. The precision of the method is also limited by the total amount of apoprotein that can be loaded onto each gel. Although in our studies 40–80 μg of apoC-III was generally isolated from each timed sample, the affinity column method allows one to obtain greater amounts for counting by simply using a larger volume of gel. This would prove desirable in those situations in which the specific activity is low: *1*) the protein cannot be labeled to a high specific activity initially; *2*) the protein is diluted by an exceptionally large unlabeled plasma pool; or *3*) samples are analyzed from later time points when the majority of the tracer has been cleared. Although the isoelectric focusing method does offer the advantage of separating the individual isoforms of apoC-III, this does not appear to be essential; the data of Huff et al. (15) indicate that the FCR for apoC-III_1 and apoC-III_2 are identical within each subject.

Biphasic decay of apoC-III specific activity was observed in all subjects. The more rapid first component had a half-life of approximately 2–3 hr and required frequent early sampling to characterize its decay. In hypertriglyceridemic subjects, the decay of 80% of the injected apoC-III tracer is described by the second and slower component (half-life greater than 20 hr). In the individuals with normal lipid levels, however, this slow component accounted for the decay of only 40–53% of the injected dose (Table 2). In the previous study by Huff et al. (15), curves containing a single exponential were found to describe the data set for the majority of subjects in both the normal and hypertriglyceridemic groups. While the nature of these two rates is not clear, it should be noted that the values obtained in our studies for the half-life of the slow "second" component correspond to values estimated by these latter investigators for their monoexponential curves (22.5 vs. 28 hr for normal and 45.1 vs. 57 hr for hypertriglyceridemic subjects). The failure to detect an early component in this previous work may be explained by the few measure-

ments of VLDL apoC-III specific activity during the first 6 hr following injection of the tracer. Thus it is not surprising that the FCRs calculated from the data in our normal subjects differ significantly from that of Huff et al. (15). For individuals with normal levels of plasma triglyceride, we estimated the FCR to be 0.040 and 0.058 hr^{-1}, compared to their reported mean value of 0.027 hr^{-1} (range 0.016–0.040) following analysis of the decay curves using a single exponential term. For subjects with plasma triglyceride levels greater than 200 mg/dl, Huff et al. (15) reported a mean FCR for apoC-III of 0.013 hr^{-1} (range 0.008–0.018); the estimated FCRs in our two subjects with hypertriglyceridemia were 0.016 and 0.022 hr^{-1}. As noted earlier, the contribution of the first component was small in these latter subjects, and hence had little effect on the calculated FCR.

It must be emphasized that these estimated values, even though derived from the use of two exponential components in the decay curve, represent only first approximations of the fractional clearance rates for VLDL apoC-III. A closer examination of our data suggests that the metabolism of VLDL apoC-III is even more complex; there is evidence of fine structure in the decay curves which is not addressed by a simple curve-peeling approach. More specifically, all turnover studies completed to date have shown a systematic deviation from biexponential decay between 9 and 15 hr following the tracer injection (Fig. 4). This phenomenon is characterized by either a constant apoC-III SA or even an increasing apoC-III SA in the two or three timed samples obtained during this period. Similar observations have been reported by Berman et al. (14) who suggested that a recycling of apoC-III radioactivity from HDL back to VLDL could account for this "hump" in the decay curve. Additional kinetic data from combined VLDL and HDL tracer studies, as well as sampling from other pools (e.g., lymph and tissues) using animal models, would be desirable to provide a more complete understanding of apoC-III metabolism. We believe the affinity chromatography system provides the basic tool for such kinetic studies.

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