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PURIFICATION OF BIOLOGICALLY ACTIVE APOLIPOPROTEINS BY CHROMATOFOCUSSING

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SUMMARY

Chromatofocussing has been used to isolate homogeneous apolipoproteins (apo) from human very-low-density lipoproteins and high-density lipoproteins with protein recovery of 70%. The inclusion of sulfhydryl-reducing agent (dithiothreitol) was required during solubilization of the lipoproteins (following delipidation) to achieve reproducible elution profiles. Removal of polyvalent buffers from apoproteins was rapidly accomplished on small columns of hydroxylapatite. The biological activity of purified apo AI and apo CII was confirmed by assessment of their ability to activate lecithin:cholesterol acyltransferase or lipoprotein lipase, respectively. Functional properties of isolated apo E were assessed by in vitro interaction with the low-density lipoprotein receptor expressed by cultured fibroblasts Apolipoproteins purified by this rapid procedure exhibit identical physical, chemical and biological properties to those purified by other, more tedious techniques.

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INTRODUCTION

Investigation of the role of apolipoproteins (apo) in lipoprotein metabolism frequently utilizes model particles generated from purified apolipoproteins and defined lipids. These studies often concern the regulation of lipoprotein lipase (LPL) and lecithin:cholesterol acyltransferase (LCAT) activities by specific apolipoproteins in normal and pathologic states. These types of studies require milligram quantities of pure, biologically active proteins.

The classical approach to apolipoprotein purification has been ion-exchange chromatography, alone [1] or in combination with gel permeation chromatography [2, 3] in the presence of 8 *M* urea. Although the resolution of high-density (HDL) and very-low-density lipoprotein (VLDL) peptides is achieved by these methods, large columns and extended run times are required for large-scale separations. Furthermore, under certain conditions long-term exposure to urea can lead to carbamylation of primary amino groups possibly affecting physiological function [4]. Preparative isoelectric focussing (IEF) techniques have been successfully applied to apolipoprotein purification [5, 6] achieving rapid and high capacity separations. The introduction of carrier ampholytes, however, and/or the effort required for their removal may affect the biological activity of the purified peptides.

Chromatofocussing [7] is a relatively new technique which combines the high resolving power of IEF with the high capacity of ion-exchange column chromatography. Applications to VLDL [8—10] and HDL [11, 12] have been reported. However, considerable variations in elution profiles were observed in different laboratories. Chromatofocussing has often resulted in better recovery than preparative IEF, but the biological activity of the apolipoproteins isolated by chromatofocussing has not yet been thoroughly evaluated [13].

In this report, the purification of apoproteins by chromatofocussing and subsequent investigations of biologic activities are described. A rapid method for the removal of buffers from the apoprotein solutions is also provided. Reproducible large-scale purification of HDL and VLDL apoproteins in biologically active form can thus be achieved.

EXPERIMENTAL

Blood collection

Blood was collected from the antecubital vein into packs containing citrated dextrose (Fenwall Labs.) and plasma was separated by low-speed centrifugation at 4°C. EDTA and sodium azide were added to 1 m*M* and 0.03%, respectively.

Isolation of apolipoproteins

VLDL and HDL were isolated by sequential ultracentrifugation at densities 1.006 g/ml for VLDL and 1.085—1.210 g/ml for HDL, as previously described [14]. All lipoproteins were purified by a second ultracentrifugation step at the upper density limit and were dialysed extensively against 0.15 *M* sodium chloride containing 1 m*M* EDTA and 0.03% sodium azide. Total protein was measured by the method of Lowry et al. [15] using bovine serum albumin (BSA) as standard and chloroform extraction to remove turbidity [16].

Delipidation was carried out according to Scanu and Edelstein [17] with ethanol—diethyl ether (3:1) and the final pellet was dried under a stream of nitrogen and stored at −20° C until use.

Apoprotein pellets were solubilized in 10 m*M* Tris—HCl (pH 7.4) containing 7.2 *M* urea and 10 m*M* dithiothreitol (DTT). After stirring for 16 h at 4° C, insoluble apo B aggregates were removed by centrifugation (5000 *g*, 20 min) and the supernatant adjusted to 10 mg/ml protein with solubilizing buffer.

All urea-containing solutions were prepared from twice deionized (Rexyn I300, Fisher Scientific) 8 *M* urea, stored at 4° C and used within 48 h of preparation. Chromatographic procedures were performed at 4° C.

Column chromatography

Chromatofocussing. Samples (10—100 mg) of apoprotein were fractionated on columns of PBE 94 (Pharmacia Canada) equilibrated with 25 m*M* imidazole · HCl (pH 7.4)—1 m*M* EDTA—7.2 *M* urea. Column dimensions were adjusted to suit the protein load, 20 × 1.0 cm for 10—30 mg and 70 × 1.5 cm for 30—100 mg. The pH gradient was formed by elution with Polybuffer 74 pH 4.0 diluted 1:8 with 8 *M* urea. Fractions of 2—5 ml were collected at a flow-rate of 25 ml/h and absorbance at 280 nm (A_{280}) and pH determined. Approximately 300 ml were required to complete the pH gradient on the smaller column and 1000 ml on the larger. Individual peaks were pooled and Polybuffer removed (as described below) prior to analysis and storage.

Removal of Polybuffer. For a protein fraction of 10 mg, 2 g of Biogel HTP (Bio-Rad Labs. Canada) were equilibrated with 10 m*M* sodium phosphate (pH 7.2) containing 0.1% sodium dodecyl sulfate (SDS) and poured into a small disposable column (10 × 1.5 cm). The chromatofocussing sample (containing 7.2 *M* urea) was applied directly to the HTP column. Following a 100-ml wash with 10 m*M* phosphate—0.1% SDS, protein was eluted with 0.5 *M* phosphate (pH 6.8)—0.1% SDS and monitored by A_{280}. Fractions containing absorbing material were recovered and dialysed against 150 m*M* sodium chloride— 1 m*M* EDTA—0.03% azide, initially at room temperature, followed by two changes of dialysate at 4° C.

Gel chromatography. Aliquots (5—15 mg) of apoprotein (volume 1.5—3.0 ml) were fractionated on a 60 × 2.6 cm column of Sephacryl S-200 (Pharmacia Canada) equilibrated with 0.1 *M* Tris—HCl (pH 7.4) containing 1 m*M* EDTA and 7.2 *M* urea. Fractions (5 ml) of the eluent were collected and A_{280} monitored. Peak fractions were pooled and dialysed against 0.15 *M* sodium chloride–1 m*M* EDTA—0.03% sodium azide. Following concentration to approximately 1 mg/ml (Amicon PM10 or YM2 membrane) the peptides were identified by SDS polyacrylamide gel electrophoresis (PAGE) or IEF and stored at −20° C.

Analytical procedures

Apolipoprotein mass was determined by the Lowry method following trichloroacetic acid (TCA) precipitation as described by Peterson [18]. Polybuffer contamination was assessed by comparison of the color yield with and without TCA precipitation. A standard curve, constructed with diluted Polybuffer, was used to estimate contamination of the apoprotein samples.

Discontinuous SDS-PAGE [19] was performed in 0.75-mm slabs of acrylamide. Analytical IEF in 5-mm cylindrical gels was performed by the method of Warnick et al. [20].

Assessment of biological activity

LCAT activation. The ability of purified apo AI to activate LCAT was assessed using egg yolk phosphatidylcholine:cholesterol liposome substrates (4:1 molar ratio) prepared according to Batzri and Korn [21]. Each assay contained 23.25 n*M* [^{3}H]cholesterol (0.03 μCi/nmol), 2% (w/v) fatty acid-free BSA, 10 m*M* Tris—HCl (pH 7.4), 150 m*M* sodium chloride, 5 m*M* EDTA and 10 m*M* 2-mercaptoethanol in a final volume of 0.2 ml. Apoproteins and liposome substrates were preincubated for 30 min at 37°C prior to the addition of purified LCAT [22]. After incubation for 30 min, the reaction was terminated by the addition of 4 ml of chloroform—methanol (2:1) and esterification rate determined as described previously [23]. The amount of LCAT added was adjusted to ensure that all experiments were carried out with linear reaction rates up to maximum apo AI activation.

Functional properties of apo AII were determined by its addition at the apoprotein/liposome preincubation stage to test the ability of this apoprotein to inhibit AI activation.

Activation of bovine milk lipase. Lipase activity was purified from bovine skim milk by heparin-agarose chromatography as described by Matsuoka et al. [24]. Substrates for measurement of triglyceride hydrolysis rate were prepared according to Baginsky [25] and preincubated for 15 min at 37°C in the presence of apoprotein prior to the addition of LPL to initiate the reaction. Each assay (final volume 0.5 ml) contained 0.14 *M* Tris—HCl (pH 8.2), 0.06 *M* sodium chloride, 5% (w/v) fatty acid-free BSA and 1.5—9 m*M* [^{3}H]glycerol trioleate (0.11 μCi/μmol). After 20 min incubation at 37°C, the reaction was stopped by the addition of acidified isopropanol and the release of [^{3}H]oleic acid determined according to Schotz et al. [26]. [^{14}C]Oleic acid was included in each assay series to determine extraction efficiency. Under these conditions less than 20% of the glycerol trioleate was hydrolysed during the incubation period and the initial rates of hydrolysis were linear for all concentrations tested.

Receptor competition studies. The biological activity of the purified apo E was assessed by determining its ability to compete for the low-density lipoprotein (LDL) receptor of cultured human fibroblasts. Apo E was isolated by chromatofocussing from the VLDL of hypertriglyceridemic patients with apo $E_{3/4}$ and $E_{2/2}$ phenotypes. Normal human skin fibroblasts were seeded in 35-mm culture dishes at a density of $5 \cdot 10^4$ cells per dish in Dulbecco's minimal essential medium (MEM) containing 10% fetal calf serum (FCS). The cells were fed after three days with the same medium. After six days growth in 10% FCS-containing medium, the dishes were washed free of FCS and replaced with MEM containing 10% lipoprotein-depleted fetal calf serum (LPDS) for 48 h incubation. Binding studies were performed as described by Rall, Jr. et al. [27] using apo E complexes with dimyristoyl phosphatidylcholine (DMPC) prepared according to Roth et al. [28] except that reisolation by gradient ultracentrifugation was omitted. Complexes were

prepared the day before the binding study and were stored at room temperature at 0.75 mg/ml protein (apo E—DMPC = 2.67:1, w/w). LPDS-treated cells were washed at room temperature with Dulbecco's phosphate-buffered saline (PBS) and incubation medium was added. This contained 1% LPDS, 2 μg/ml [^{125}I] LDL (350 cpm/ng) and the indicated concentration of apo E—DMPC complex. The dishes were then cooled on ice and incubated for 2 h at 0—4° C. At the conclusion the cells were washed and cell-associated LDL determined by previously published methods [29].

RESULTS

Purification of apoproteins by chromatofocussing

The chromatofocussing column profile of normal apo HDL is shown in Fig. 1. Three major peaks were observed at pH values of 8.3, 5.95 and 5.66. These were identified as apo CI, AII and AI, respectively, by SDS-PAGE and IEF. The fractions were homogeneous (Fig. 2) as judged by electrophoretic criteria. The elution pattern was reproducible up to an initial load of 100 mg of apoprotein, provided the sample was treated with DTT as indicated in Experimental. Extending the time between solubilization and column loading caused cross-contamination of apo AI and AII (data not shown). This is likely to be due to differences in the isoelectric point (p*I*) of the monomer and dimer forms of apo AII. In the dimer form AII appears to co-elute with apo AI during chromato-focussing. Dimerization apparently occurs in the absence of sulfhydryl-reducing agents. The apo CI peak represented approximately 7% of applied apo HDL and was identified by its low molecular weight on SDS-PAGE and the absence of protein in the pH range 4—6 on IEF. A small component, approximately 7%

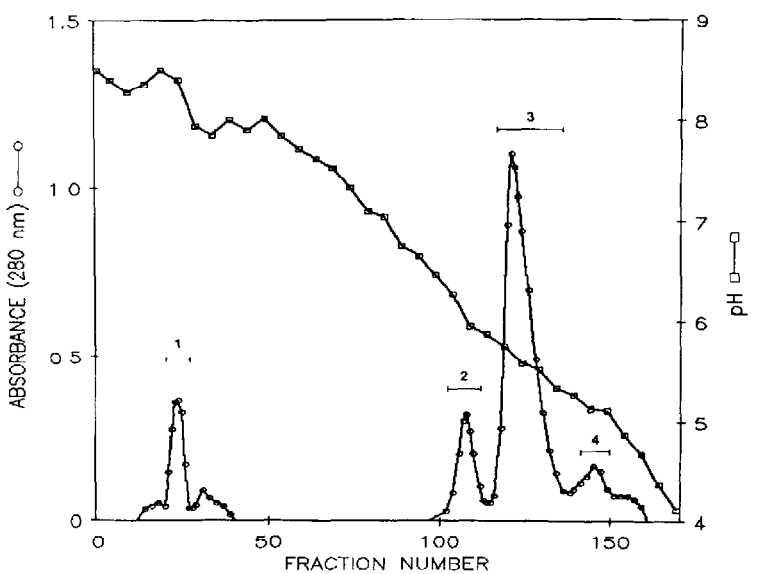

Fig. 1. Chromatofocussing column profile of normolipidemic HDL apoproteins. Apoprotein (90 mg) was applied and 5-ml fractions were collected at a flow-rate of 25 ml/h from a 70 × 1.5 cm column. Numbers indicate pooled regions of homogeneous apoprotein species. 1 = apo CI; 2 = apo AII; 3 = apo AI; 4 = apo AI—AII mixture

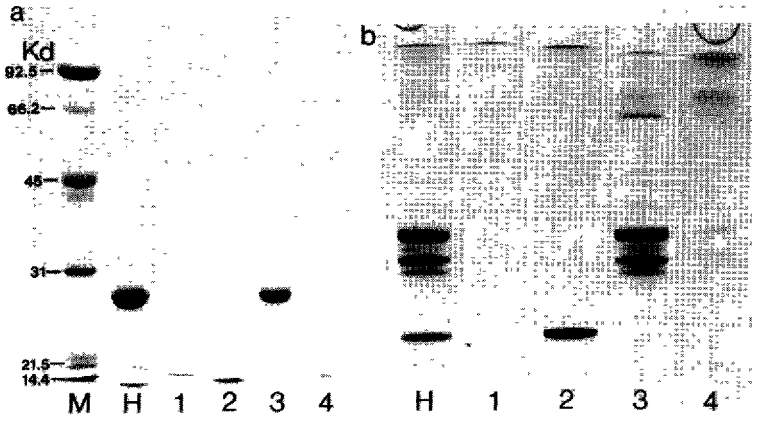

Fig. 2. Identification of HDL apoproteins isolated by chromatofocussing. (a) SDS-PAGE gel of HDL apolipoproteins in 10% polyacrylamide. M = molecular mass markers; H = total HDL apoproteins, numbers 1—4 indicate pooled fractions from Fig. 1. (b) IEF gels of HDL apolipoproteins in pH 4—6 gradient, 7.5% acrylamide. Labeling scheme is same as in (a).

of the total HDL protein, contained both apo AI and AII and eluted after the major apo AI peak. Less than 5% of the protein loaded was eluted with 1 *M* sodium chloride.

The removal of amphoteric buffers by precipitation, dialysis and gel filtration has been incomplete in our hands. However, the use of hydroxylapatite chromatography removed more than 99% of these compounds from apolipoprotein preparations. The recovery of protein from the apo HDL was 75% after chromatofocussing, HTP treatment and dialysis.

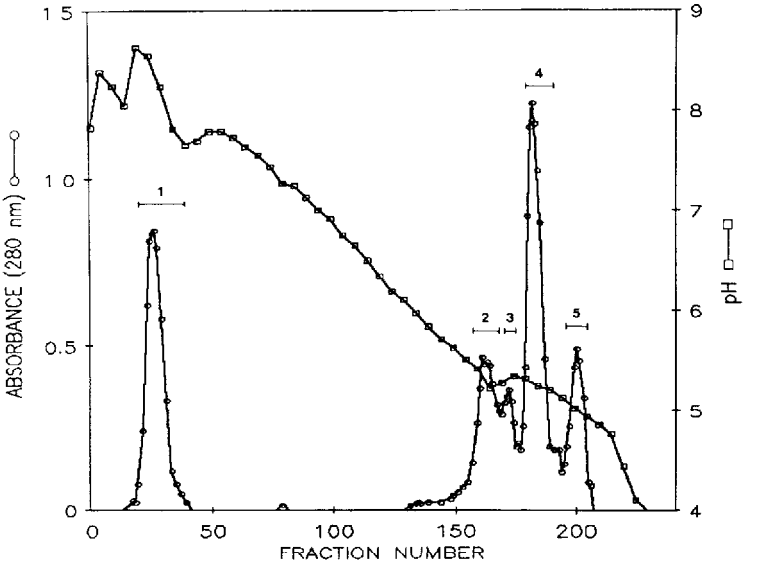

Fig. 3. Chromatofocussing column profile of VLDL apoproteins. Urea-soluble fraction (100 mg) was applied to a 70 × 1.5 cm column and 5-ml fractions were collected at 25 ml/h. Numbers indicate fractions pooled as homogeneous species. 1 = apo CI, 2 = apo E; 3 = apo CII, 4 = apo CIII-1; 5 = apo CIII-2.

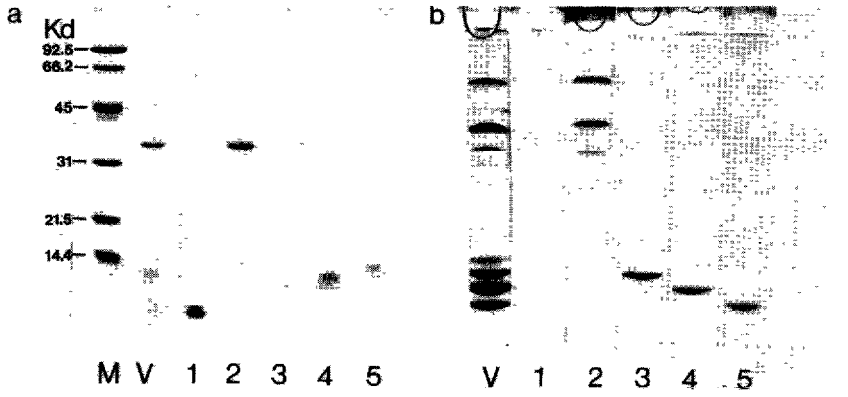

Fig. 4. Identification of VLDL apoproteins isolated by chromatofocussing. (a) SDS-PAGE gel electrophoresis of VLDL apoproteins in 12.5% acrylamide. M = molecular weight markers; V = total apo VLDL; numbers refer to pooled fractions from Fig. 3. (b) IEF gels of VLDL apoproteins in pH 4—6 gradient, 7.5% polyacrylamide. Lane contents are as indicated in (a).

A chromatogram of apo VLDL purification on PBE 94 is shown in Fig. 3. This profile was obtained using VLDL from a patient with Type V hyperlipidemia and the apo $E_{3/4}$ phenotype. Peaks were identified as indicated in Fig. 4. The apo E peak showed a marked shift in p*I* from its position observed on IEF gels (Fig. 4b) resulting in co-elution of apo E, CIII-0 and CII. Similar results were obtained with VLDL isolated from subjects with other apo E phenotypes. Subsequent chromatography on Sephacryl S-200 resolved apo E from apo C components (Fig. 5). The recovery of protein material from the urea-soluble component of VLDL after these procedures was approximately 70%.

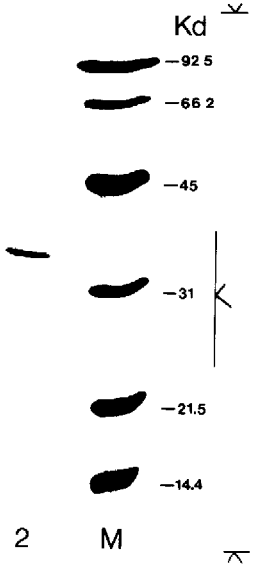

Fig. 5. Comparison of apo E species purified by chromatofocussing from different apo E phenotypes. Electrophoresis was performed in 12.5% acrylamide in the presence of SDS and 2-mercaptoethanol. M = molecular weight markers, 1 = apo $E_{2/2}$; 2 = apo $E_{4/3}$. Each lane contains 20 μg protein.

TABLE I

COMPARISON OF ISOELECTRIC POINTS OBTAINED BY IEF AND CHROMATOFOCUSSING

Results for IEF are from the references indicated in brackets and those for chromatofocussing from Figs. 1 and 3.

Apoprotein	Isoelectric point	
	IEF	Chromatofocussing
AI [6]	5.30, 5.42	5.66
AII [6]	4.8	5.95
CI [5]	>6.5	8.4
CII [5, 10]	4.79—5.01	5.30
CIII-1 [5, 10]	4.70—4 91	5.28
CIII-2 [5, 10]	4 50—4.80	5.00
E [5, 10]	5.6—6.2	5.33

Previous work has indicated discrepancies in the p*I* values determined by chromatofocussing and those obtained by analytical IEF. Our data also indicate that this is the case (Table I). Elution from PBE columns is dependent on buffering capacity of the functional groups on the gel matrix and on homogeneity of eluting species in the mobile phase. The differing distribution of functional groups in the eluent buffer compared with ampholines used for analytical IEF may be responsible for the discrepancies in p*I* values between the two methods.

Assessment of biological activity

LCAT activation. The marked enhancement of cholesterol esterification by purified LCAT on the addition of apo AI is indicated in Fig. 6. Optimal activation was observed at an AI concentration of 25 μg/ml (0.9 μ*M*), similar to that previously reported [30]. At this molar concentration activation by apo AII, E, CI, CII, CIII-1 and CIII-2 was less than 10% of the activation by apo AI. However, by increasing the apoprotein concentration further, significant activation could be demonstrated for apo E and CI (Fig. 7). Using 0.9 μ*M* apo AI as 100% activation, maximal activation of LCAT was 64% at 7.5 μ*M* apo E and 57% at 16 μ*M* apo CI. Apo CI activation has been described by Albers et al. [30]. Recently, Chen and Albers [31] and Zorich et al. [32] have also shown that apo E is a significant LCAT activator. Apo AII purified by chromatofocussing produced inhibition of apo AI activation as shown in Fig. 8.

Bovine milk lipase activation. The ability of isolated apo CII to activate lipoprotein lipase was tested using the bovine milk lipase as a model system. This enzyme has similar properties to the enzyme purified from human post-heparin plasma [33]. Double reciprocal plots at four levels of triglyceride are shown in Fig. 9. From these data an apparent Michaelis constant, K_m (CII) value of $2.5 \cdot 10^{-7}$ *M* was calculated at all trioleylglycerol concentrations tested. This result agrees well with the data of Posner et al. [34] who calculated a K_m value of $2.5 \cdot 10^{-8}$ *M* using a similar system.

Receptor competition studies. Investigation of apo E phenotypes over the

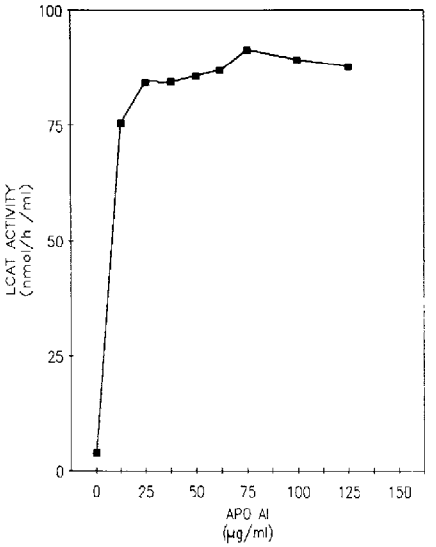

Fig. 6. Activation of LCAT by apo AI purified by chromatofocussing. Each point represents the mean of duplicate incubations differing by less than 5%.

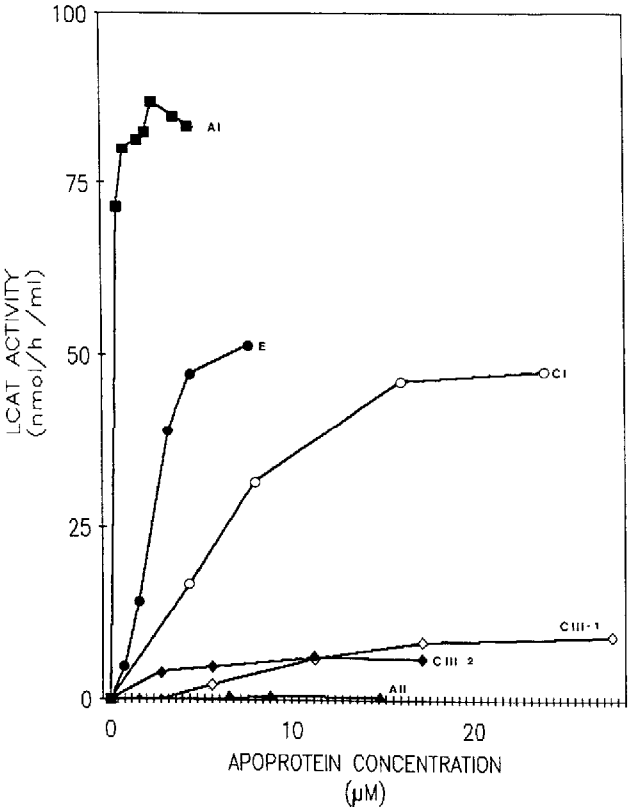

Fig. 7. Concentration dependence of LCAT activation for apoproteins of HDL and VLDL. Results are expressed in terms of molar apoprotein concentration for easier comparison. Each data point is the mean of duplicate incubations.

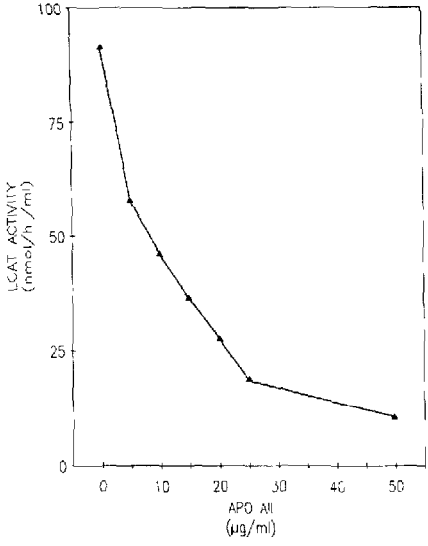

Fig. 8. Effect of apo AII on apo AI-mediated LCAT activation. Apo AII was purified by chromatofocussing and added to LCAT assay mixtures containing optimal amounts of apo AI for LCAT activation (75 μg/ml). Results are means of duplicate incubations.

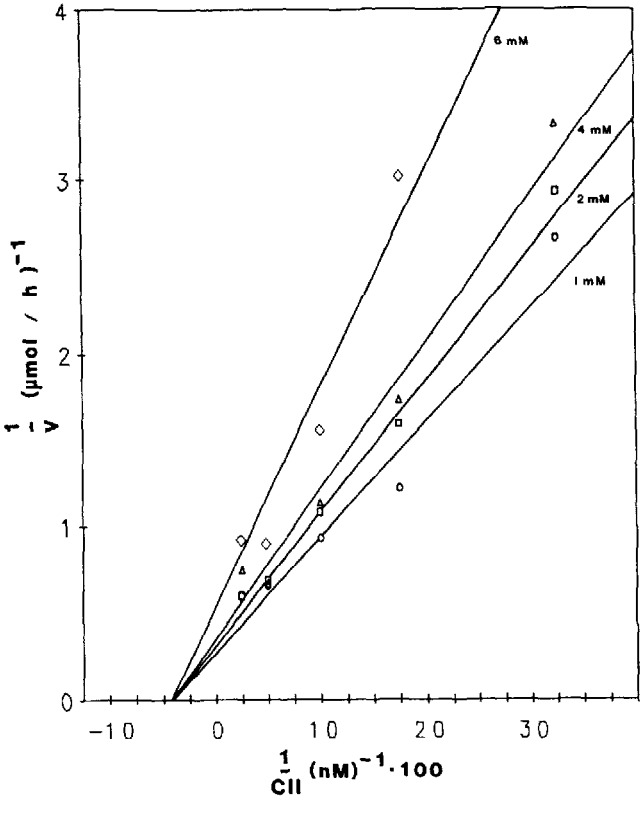

Fig. 9. Activation of bovine milk lipase by apo CII purified by chromatofocussing. The figure shows double reciprocal plots of triglyceride hydrolysis as a function of apo CII concentration. Triglyceride hydrolysis by LPL at 1.0 m*M* (○), 2.0 m*M* (□), 4.0 m*M* (△) and 6.0 m*M* (◇) triolein were measured. Each point represents the mean of duplicate assays.

last several years has revealed the role of specific amino acid residues in the interaction of apo E with the LDL or apo B/E receptor on cultured cells [35]. Individuals with the apo $E_{2/2}$ phenotype present with the Type III hyperlipidemia [36] due to the impairment of these receptor interactions. Isolation of apo E from these subjects and recombination with phospholipid represents a method by which one can investigate the interaction of this apoprotein with the LDL receptor of skin fibroblasts. Innerarity et al. [29] have shown that apo E_2 is not as effective at displacing LDL from these cells as apo E_3 or E_4. Fig. 10 shows such an experiment using total apo E isolated from two hyperlipidemic subjects by chromatofocussing, one with phenotype apo $E_{3/4}$ and one with phenotype apo $E_{2/2}$. LDL, apo E_2 complexes and DMPC vesicles alone show approximately the same capability to displace labelled LDL from the cells with a maximal displacement of approximately 25% of the label. Apo $E_{3/4}$ complexes were much more effective, as reported [27], with more than 50% displacement at a ligand concentration of 1 μg/ml.

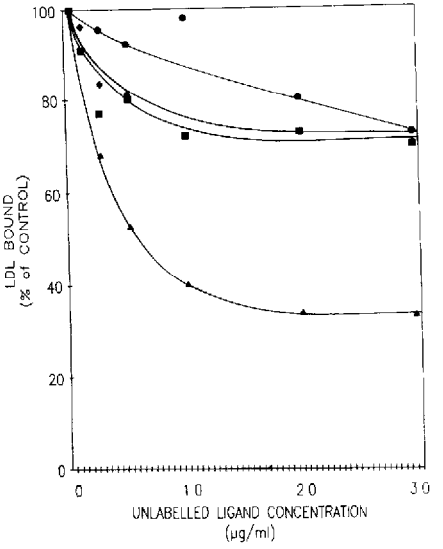

Fig. 10. Displacement of LDL by dimyristoyl phosphatidylcholine (DMPC) liposomes containing apoprotein E purified by chromatofocussing. Fibroblasts were grown in 35-mm culture dishes for six days in the presence of 10% FCS after which medium containing LPDS was substituted. After 48 h incubation dishes were washed free of medium and competition studies performed. Medium was added containing 2 μg/ml LDL (350 cpm/ng) and the indicated concentration of DMPC liposomes. Cell-associated radioactivity was measured after 2 h incubation at 4°C. (♦) Liposomes without apo E; (▲) liposomes with apo $E_{3/4}$; (■) liposomes with apo $E_{2/2}$. The displacement obtained by LDL is also shown for comparison (●). All points are mean values for duplicate dishes.

DISCUSSION

Purification of apoproteins by chromatofocussing has been reported from several laboratories [8—13]. The data presented here indicate that this technique is a viable approach to the single-step purification of all of the apolipoproteins of VLDL and HDL, with the possible exception of apo E and

CII. The latter polypeptides may be further resolved by gel chromatography. The removal of polybuffers can be efficiently accomplished by the use of hydroxylapatite columns to less than 0.1% of the original concentration. The overall procedure is rapid and the products are sufficiently concentrated for analysis without further manipulation. The combined yield of apoproteins is of the order of 70% which is superior to most (if not all) other techniques. The biologic activity of the isolated apoproteins has been assessed by enzyme activation and receptor interaction studies. Our findings confirm previously published observations in this area for apolipoproteins purified by other techniques.

The discrepancies in column profiles, indicated in previous work, are overcome by resolubilizing the delipidated apoproteins in the presence of a sulfhydryl-reducing agent. Our results compare very closely with those of Marz and Grob [10] who have used the same protocol as described here for VLDL. Techniques for isolation of HDL apoproteins [11, 12] have not uniformly used sulfhydryl-reducing agents and these publications report apo AI contamination of all fractions. In this regard, the apo AII monomer appears to be much more readily separated from apo AI than its dimerized form which occurs in the absence of DTT.

In summary, we feel that chromatofocussing is a simple and reproducible technique for the purification of nearly all apoplipoproteins. The advantages over other methods are its isocratic nature and its high resolution with relatively high flow-rate. The method is applicable to both HDL and VLDL and relatively small columns can fractionate up to 100 mg of protein. The proteins are easily separated from buffering species on hydroxylapatite and are biologically active as assessed by LCAT- and LPL-activating ability and by receptor interaction. A single large-scale purification method such as this is extremely valuable for assessment of further functional roles of the specific apolipoproteins in model lipoprotein systems.

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