Journal of Chromatography, 342 (1985) 47—66
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2594

EVALUATION OF IMMUNOAFFINITY CHROMATOGRAPHY FOR ISOLATING HUMAN LIPOPROTEINS CONTAINING APOLIPOPROTEIN B

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(Received December 18th, 1984)

SUMMARY

Because of high specificity, immunoaffinity chromatography is the most suitable procedure for the isolation of lipoprotein (LP) particles defined by their apolipoprotein (Apo) composition. The purpose of the present study was to describe immunosorber methodology and its application to the isolation of ApoB-containing lipoproteins from either plasma or isolated lipoprotein density classes. The exploration of various coupling procedures demonstrated that immunosorbers of highest capacity were obtained by cyanogen bromide activation of Sepharose. Among various dissociating agents tested, 3 *M* sodium thiocyanate was found to be the most effective desorbent for bound lipoproteins. Studies on the non-specific binding of serum albumin to several different immunosorbers showed a negligible retention (1.9%) of albumin. Good recoveries (80—98%) were obtained with all apolipoproteins tested with both anti-ApoA-I and anti-LP-B immunosorbers.

By using the optimal experimental conditions, it was shown that the ApoB-containing lipoproteins retained by immunosorbers with antibodies to LP-B had chemical, physical and immunological properties similar, if not identical, to those of their corresponding parent density classes. The application of an alternative immunoaffinity chromatography procedure with serially connected immunosorbers with antibodies to apolipoproteins other than ApoB resulted in the isolation of LP-B, a lipoprotein containing ApoB as its sole protein constitutent. LP-B had chemical and physical properties very similar to those of subclass 2 of low-density lipoproteins (density 1.019—1.063 g/ml, flotation coefficient 0—12). Based on these studies, we suggest that immunoaffinity chromatography in combination with microanalytical procedures for quantification of lipids and apolipoproteins offers a powerful tool for the isolation and functional characterizaton of lipoprotein particles defined by their apolipoprotein composition.

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0378-4347/85/$03.30

INTRODUCTION*

The presence of a number of apolipoproteins and their wide distribution across the density spectrum have added a new dimension to the complexity of human plasma lipoproteins [1—3]. Operational definitions and classifications of plasma lipoproteins have recognized the heterogeneity of plasma lipoproteins with respect to size, hydrated density and electrical charge and provided a conceptual framework for studies of their physicochemical properties and role in lipid transport [4]. However, owing to inherent physical rather than chemical criteria, operational classification systems cannot account for the protein heterogeneity of plasma lipoproteins.

Results of immunological characterization and apolipoprotein quantification of major lipoprotein density classes have shown clearly that each segment of the density spectrum consists of several qualitatively distinct lipoprotein particles rather than single homogenous lipid—protein complexes [3, 5]. In other words, the operationally defined lipoproteins appear to be mixtures of lipoprotein particles characterized by similar hydrated densities or electrical charges but different apolipoprotein compositions. Isolation of distinct LP-X, LP-A and LP-B particles from LDL of patients with obstructive jaundice represented the first successful isolation of lipoproteins defined by their apolipoprotein composition [6]. Several ensuing studies on the isolation of simple and complex lipoprotein particles have provided additional evidence for the occurrence of discrete lipoprotein particles [7—13].

Since apolipoproteins can replace physical properties as a criterion for recognizing and differentiating lipoproteins, we and other investigators [14—20] have exploited the specificity of antibodies to explore immunoprecipitation and immunoaffinity chromatography as procedures for the isolation and purification of lipoprotein particles. In searching for the most suitable method for the fractionation and isolation of simple and complex lipoproteins, we have primarily focused our attention on immunoaffinity chromatography. The present report describes the preparation of immunosorbers specific for ApoB and their application to the isolation of lipoproteins which contain ApoB either as their sole protein (simple lipoproteins) or as one

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*Nomenclature and abbreviations: Chylomicrons (flotation coefficient, $S_f > 400$); VLDL = very-low-density lipoproteins (density, $d < 1.006$ g/ml, $S_f > 20$); LDL_1 = subclass of low-density lipoproteins (d 1.006—1.019 g/ml, S_f 12—20); LDL_2 = subclass of low-density lipoproteins (d 1.019—1.063 g/ml, S_f 0—12); HDL = high-density lipoproteins (d 1.063—1.21 g/ml). Apolipoproteins A-I, A-II, B, C-I, C-II, C-III, D, E and F are referred to as ApoA-I, ApoA-II, ApoB, etc. Simple and complex lipoproteins are named according to their apolipoprotein composition. Simple lipoproteins contain a single apolipoprotein and are named according to their apolipoprotein: LP-A-I = lipoprotein A-I, LP-B = lipoprotein B; etc. Complex lipoproteins consist of more than one apolipoprotein and are designated accordingly: LP-A (LP-A-I:A-II) = lipoprotein containing apolipoproteins A-I and A-II; LP-B:C (LP-B:C-I:C-II:C-III) = lipoprotein containing apolipoproteins B, C-I, C-II and C-III; etc. Immunosorbers are designated according to specificity: anti-LP-B immunosorber = immunosorber with covalently linked antibodies to LP-B. Retained (R) and unretained (U) fractions are designated according to their elution from specific immunosorbers in the following manner: a-ApoBU = unretained fraction from anti-LP-B immunosorber; a-ApoBR = retained fraction from anti-LP-B immunosorber

of their several protein constituents (complex lipoproteins). Since our results showed that there was no apparent effect on the physical, chemical and immunological properties of these isolated lipoproteins, we suggest that immunoaffinity chromatography represents the method of choice for the isolation of lipoprotein particles defined by their apolipoprotein constituents either directly from plasma or lipoprotein fractions derived from plasma by other procedures.

EXPERIMENTAL

Plasma

Plasma was collected after an overnight fast from both normolipidemic and hyperlipidemic subjects. Subjects were classified according to the criteria of the Lipid Research Clinics [21] as either normolipidemic or hyperlipoproteinemic.

Isolation of lipoprotein density classes

Chylomicrons ($S_f > 400$), VLDL (S_f 20—400), VLDL + LDL_1 ($S_f > 12$), LDL_2 (S_f 0—12) and HDL were isolated by preparative ultracentrifugation as previously described [1]. When required for particular studies, lipoprotein density classes were centrifuged again under the identical isolation conditions until free of albumin as determined by double-diffusion analyses. The samples were dialyzed against 0.05 *M* Tris—HCl (pH 7.5) buffer containing 0.9% sodium chloride, 0.01% EDTA and 0.01% sodium azide and utilized for fractionation studies with immunosorbers.

Immunological analyses

Double-diffusion and immunoelectrophoretic analyses were carried out as previously described [1]. Antisera to human apolipoproteins A-I, A-II, C-I, C-II, C-III, D, E, and to LP-B and immunoassays for each apolipoprotein have been described [22—27]. Double-diffusion analyses were performed on both 1% agarose and 1% Bactoagar with Bactoagar found to be a more sensitive detection system. The crossed immunoelectrophoretic technique, a two-dimensional method combining electrophoresis and electroimmunoassay, was adapted as described by Ganrot [28].

Preparation of antibodies for coupling

Totally delipidized HDL (ApoHDL) and LDL_2 were coupled to cyanogen bromide activated Sepharose 4B-CL at pH 8.0 as described by Cuatrecasas [29]. To insure a longer lifetime of the LDL_2 covalently bound to Sepharose, the LDL_2—Sepharose was cross-linked with glutaraldehyde [30]. Such a treatment allowed the repeated recycling of this antigen–Sepharose column for the isolation of antibodies to LP-B. Specific antibodies to apolipoproteins A-I and B were isolated by passing each antiserum over the ApoHDL–Sepharose and LDL_2—Sepharose columns, respectively. To separate the specific antibodies from the dissociation agent, 3 *M* sodium thiocyanate, each antigen—Sepharose column was prepared with Sephadex G-25 at the lower end of the column as previously described [10]. For some experiments, the immunoglobulin (Ig) G

fractions of antisera to LP-B, ApoC, ApoD and ApoE were prepared by precipitation with 33% ammonium sulfate. Antibodies isolated by affinity chromatography were stable in a saturated sucrose solution or in a precipitated form (50% ammonium sulfate). Stored in this manner, affinity purified antibodies can be accumulated for the preparation of immunosorbers of larger capacity. This is particularly advantageous when only small quantities of antigen are available to construct an antigen–Sepharose column.

Coupling of antibodies to solid-state matrices

To establish a procedure which would result in maximal binding of antigens to immunosorbers, several activation and coupling procedures were examined including the use of N-hydroxysuccinimide (N-Succ) esters of agarose [31], 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) cross-linking with carboxymethyl agarose [32], periodate-oxidized Sephadex [33], 1,1-carboxyldiimidazole (CDI) [34], cyanogen bromide activated Sepharose [35], and cross-linking using Sephacryl and glutaraldehyde [36]. The coupled antibody preparation was a 33% ammonium sulphate IgG fraction of an antiserum specific for LP-B. Results of these experiments led to further investigation of the cyanogen bromide coupling procedure described by Cuatrecasas [29] and March et al. [35]. Affinity-purified antibodies to LP-B were coupled to Sepharose 4B-CL using three different cyanogen bromide concentrations for activation (50, 34 and 16 mg/ml Sepharose). Equal aliquots of affinity-purified antibodies to LP-B were added at pH 7.0, 7.5 and 8.0 to equal aliquots of each activated Sepharose resulting in nine different immunosorber combinations of varying pH and degree of Sepharose activation. After the coupling of antibodies, the immunosorbers were incubated for 1 h with 0.1 *M* ethanolamine, pH 8.0, washed with 3 *M* sodium thiocyanate and equilibrated with the eluting buffer 0.05 *M* Tris–HCl, 0.15 *M* sodium chloride and 0.01% sodium azide, pH 7.5.

Dissociation of antigens bound to immunosorbers

A number of different elution buffers were tested for their capability to dissociate antigens retained on immunosorbers including low and high pH and several different chaotropic agents.

Construction of immunosorber

Since the dissociation of antigen–antibody complexes requires treatment with agents that may cause either conformational changes and/or changes in the charge of the antibody–antigen complex, it was necessary to minimize permanent alterations in the tertiary and quaternary structure of lipoproteins. One way to minimize the time of contact between the antigen and the dissociating agent in the Sepharose-based column system is to construct immunosorbers with a layer of G-25 below the immunosorber portion [10]. The Sephadex G-25 layer is three times the bed volume of the antibody–Sepharose and serves essentially as a desalting column by separating the lipoproteins from the dissociating agent. Since the entire column acts as a molecular sieve, the lipoproteins are only briefly in contact with sodium thiocyanate, thus minimizing the potentially disruptive effect of this agent on lipoprotein structure.

Non-specific adsorption

Several different approaches have been used to minimize the non-specific adsorption of plasma proteins. The first one involves the use of non-immune IgG, prepared from the same animal species as the antiserum, coupled to Sepharose. The retained fraction from an immunosorber specific for a particular apolipoprotein is passed over the non-immune IgG—Sepharose column to remove any non-specifically adsorbed proteins. The second approach utilizes antibodies coupled to Sepharose which are directed towards plasma proteins not associated with the particular apolipoprotein in question. This approach has been described for the isolation of ApoF-containing lipoproteins [12]. To eliminate non-specific adsorption caused by weak hydrophobic or ionic interactions, the immunosorber can be washed with 10 m*M* Tris, pH 7.5, or 0.5 *M* sodium chloride prior to elution of the retained fraction. Alternatively, the retained fractions from immunosorbers can be further purified by standard lipoprotein fractionation steps such as ultracentrifugation [1] or molecular sieve chromatography [37].

Concentration of samples

Several different procedures for concentrating lipoproteins have been used including the use of hygroscopic solids such as sucrose or polyethylene glycol (molecular weight, MW = 18 500) placed outside a dialysis bag (5000 MW cut-off), the direct addition of Sephadex G-25 to the sample, the use of Amicon Diaflo cells with membranes of particular molecular weight exclusion limits, and preparative ultracentrifugation.

Operation of immunosorbers

Sepharose-based immunosorbers have been primarily used in the column procedure. The immunosorbers were constructed as previously described and their capacity was determined by adsorbing whole plasma. The column chromatography was carried out at 6°C and monitored at 280 nm. An appropriate volume of the sample was placed on the immunosorber and eluted with the

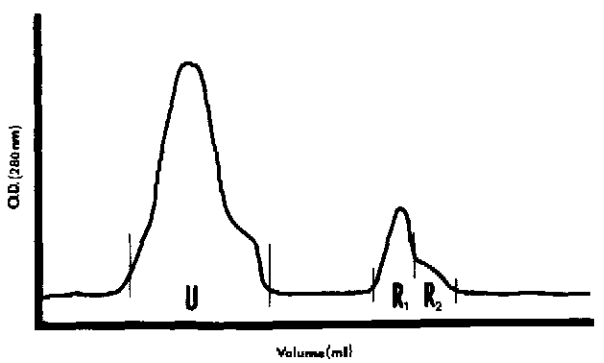

Fig. 1. Elution profile of a typical immunosorber. Immunosorber was equilibrated with 50 m*M* Tris—HCl buffer (pH 8.0) containing 0.9% sodium chloride, 0.01% EDTA and 0.01% sodium azide. U = Unretained fraction; R_1 = retained fraction not containing sodium thiocyanate; R_2 = retained fraction containing sodium thiocyanate. Sodium thiocyanate (3 *M*) was applied as dissociating agent after the absorbance of the unretained fraction returned to baseline.

equilibration buffer, 50 m*M* Tris—HCl (pH 7.5), 0.9% sodium chloride, 0.01% EDTA and 0.01% sodium azide. After the unretained fraction was eluted and the absorbance returned to baseline, 3 *M* sodium thiocyanate (20—30 ml) was applied, followed by the equilibration buffer, to desorb the retained lipoproteins. The retained fractions were eluted as two major peaks with R_1 representing the major and R_2 the minor quantity of ultraviolet-absorbing material (Fig. 1). The R_1 fraction was essentially devoid of sodium thiocyanate, while R_2 contained small quantities of lipoproteins and the principal portion of sodium thiocyanate.

Analytical methods

Lipids were analyzed by either gas—liquid chromatography [38] or TH-10 analyzer methodology as described by Mills et al. [39]. Procedure used for determining flotation coefficients has been described [13]. Acidic and basic polyacrylamide gel electrophoreses (PAGE) were performed as previously described [40]. Protein was determined by the method of Lowry et al. [41] or by the protein microassay originally described by Bradford [42]. Negative staining for electron microscopy was performed using a modification [43] of the technique described by Forte et al. [44]. Prior to microscopy, lipoprotein samples were dialyzed against 0.1 *M* ammonium acetate buffer, pH 7.4. A drop of the sample was applied to a Formvar coated copper grid, excess liquid was drained off and a drop of 2% phosphotungstate, pH 7.4, was immediately added. After 1—2 min of staining the excess stain was removed with filter paper. The grids were then screened and photographed in a Hitachi H500 transmission electron microscope at primary magnification between 15 000- and 35 000-fold.

RESULTS

Capacity of immunosorbers

The capacity of immunosorbers depends on several factors including the nature of matrices, activation procedure and quality of antibodies. Since the number of steps to prepare a specific immunosorber may vary, the capacity of each newly constructed immunosorber must be established by determining the

TABLE I

COMPARISON OF DIFFERENT COUPLING PROCEDURES

Coupling procedure*	Percentage anti-LP-B IgG coupled**	Immunosorber capacity (mg ApoB per ml gel)
N-Succ	28.1	⩽0.001
CDI	24.8	0.0026
Sodium metaperiodate	41.3	0.0054
Glutaraldehyde	48.6	0.0012
Cyanogen bromide	58.8	0.056
EDAC	76.6	0.010

*See Experimental for abbreviations.

**The same anti-LP-B (IgG fraction) was used for all experiments.

amount of apolipoprotein either in the unretained or the retained fraction. We have defined the antigen binding capacity of an immunosorber as the amount of normolipidemic fasting plasma apolipoprotein retained per milliliter of immunosorber per single passage. In the present study, the capacity of each column was determined by measuring ApoB in the retained fraction by electroimmunoassay.

As shown in Table I, the immunosorber of highest capacity was obtained by cyanogen bromide activation of Sepharose. Several commercially available solid-state activated supports were also tested, but none surpassed the cyanogen bromide activated Sepharose in binding capacity. Additional experiments on the effect of varying concentrations of cyanogen bromide and varying the pH showed that the most effective coupling of active antibody was achieved at pH 8.0 and the highest cyanogen bromide concentration examined (Table II).

TABLE II

EFFECT OF VARYING CYANOGEN BROMIDE AND pH ON THE BINDING CAPACITY OF IMMUNOSORBERS

Same preparation of affinity-purified anti-LP-B (1 mg/ml of gel) was used in this study.

Cyanogen bromide concentration (mg/ml of gel)	pH	Anti-LP-B coupled* (%)	Immunosorber capacity** (mg ApoB per ml of gel)
50	8.0	90	0.86
50	7.5	85	0.76
50	7.0	78	0.65
34	8.0	81	0.78
34	7.5	77	0.65
34	7.0	74	0.61
16	8.0	68	0.45
16	7.5	66	0.33
16	7.0	57	0.28

*Determined by difference between the initial protein concentration and protein concentration of the unretained fractions.
**ApoB of retained fraction was determined by electroimmunoassay.

TABLE III

COMPARISON OF DIFFERENT DISSOCIATING AGENTS DESORBING LIPOPROTEIN B FROM ANTI-LP-B—SEPHAROSE 4B-CL

Dissociating agent	Recovered ApoB*
3 *M* sodium thiocyanate	100
50 m*M* Glycine—HCl, pH 3.2	42
Water, pH 6.0	9.5
0.7 m*M* Sodium hydroxide, pH 9.6	33
1.0 m*M* Sodium hydroxide, pH 10	58
3.0 m*M* Sodium hydroxide, pH 10.6	58

*Values are expressed as percentage recovered ApoB in comparison with that recovered by sodium thiocyanate.

Dissociating agents

A comparison of various dissociating agents showed that 3 *M* sodium thiocyanate was the most effective desorbent for lipoproteins bound to Sepharose-based immunosorbers (Table III). Furthermore, immunosorbers treated with 3 *M* sodium thiocyanate appear to retain their binding capacity to a greater degree with repeated usage than immunosorbers desorbed with glycine buffer at low pH. The dissociating capacity of 3 *M* sodium thiocyanate was greater than those of either low- or high-pH buffers. However, the use of two different dissociating agents offered the possibility to compare the effects of these agents on the physicochemical and biological properties of retained lipoproteins.

Effect of concentration

The application of solid-state immunosorbers usually yields lipoprotein fractions that have to be concentrated in order to determine their physicochemical and biological properties. We have compared two different procedures for concentrating lipoprotein solutions by measuring the recovery of ApoB and neutral lipids. One of these procedures was based on the use of dialysis tubing with either sucrose or polyethylene glycol outside the dialysis bag and the other utilized Amicon Diaflo cells with four membranes of different porosity. A sample of whole plasma was diluted twenty-fold and concentrated back to its original volume. Since the results of experiments with sucrose and polyethylene glycol were similar, they were combined for comparison with the results of the experiment with the Amicon concentration cell. These analyses indicated that both methods gave similar lipid recoveries (Table IV). The recovery of ApoB was lower than that of lipids suggesting the possible antigenic instability of this apolipoprotein [45].

Tests with the Amicon membranes showed that traces of apolipoproteins A-I, A-II and C-III could only be detected in the filtrate after the application of XM-300 membrane though the recovery of the concentrated material was similar to the reported mean values. The least satisfactory membrane was the PM 10 membrane with recoveries of about 80% of the untreated plasma. It appears that among the tested membranes, the XM-50 membrane is the most

TABLE IV

EFFECTS OF DILUTION AND CONCENTRATION ON THE RECOVERY OF PLASMA LIPIDS AND APOLIPOPROTEINS

Recovery after diluting (twenty-fold) and concentrating 5 ml of whole plasma back to its original volume.

Procedure	Percentage recovery (mean ± S.D.)					
	ApoA-I*	ApoB*	Cholesterol	Cholesterol esters	Triglyceride	Total neutral lipids**
Dialysis membrane (n = 5)	92.3 ± 1.5	72.2 ± 10.0	76.4 ± 6.7	70.9 ± 7.1	89.9 ± 15.4	76.9 ± 3.1
Diaflo membranes*** (n = 4)	91.5 ± 11.6	73.8 ± 11.2	82.3 ± 5.2	82.6 ± 5.1	85.9 ± 4.7	82.5 ± 5.6

*Apolipoproteins were determined by electroimmunoassay.
**Total neutral lipids were determined by gas–liquid chromatography.
***Four different membranes were used in an Amicon concentration cell: XM300, XM50, PM30 and PM10.

satisfactory one owing to its flow characteristics. In our experience, if a large number of samples are to be concentrated, the use of dialysis tubing combined with either polyethylene glycol or sucrose seems to be equally satisfactory. However, sucrose appears to have a better stabilizing effect on the structural integrity of lipoproteins than polyethylene glycol; a similar effect was also seen with the preparation of affinity-purified antibodies. Preparative ultracentrifugation is another useful procedure for concentrating dilute solutions resulting in recoveries of the various lipoprotein components in the 65- 90% range.

Specificity of immunosorbers and non-specific adsorption

Two other important aspects of immunosorber methodology are concerned with the specificity of immunosorbers and the possibility of non-specific adsorption of lipoprotein particles and plasma proteins other than those corresponding to the attached antibodies.

Specificity of immunosorbers is determined by at least two factors, i.e. the monospecificity of the antibodies coupled to the Sepharose and the extent of interactions of the solute with the solid phase of the immunosorber. In the present study, the antibodies coupled to the immunosorber were monospecific as judged by double-diffusion analyses and crossed immunoelectrophoresis carried out with purified apolipoproteins and whole plasma. This also applied to the affinity-purified antibodies to ApoA-I, ApoC and LP-B and the IgG fraction of antisera to ApoD and ApoE. The problem of non-specific adsorption is more difficult to assess since it could be due to ion-exchange or hydrophobic effects, interactions between lipoproteins specifically bound to the immunosorber and other lipoprotein species, and interactions with serum proteins.

Human serum albumin was a suitable protein available for testing the non-specific binding of immunosorbers because it shares with lipoproteins the capacity to bind fatty acids and lysolecithin. When applied (25 mg per aliquot) to immunosorbers specific for ApoA-I, ApoB, ApoC, ApoD and ApoE, the mean recovery of human albumin in unretained fractions was 98.1% (the range for five columns was 96.6—100%). No relationship was found between the content of albumin in the retained fraction and the type of antibodies used. These results have indicated a minimal non-specific binding of human serum albumin. In addition, the fraction of whole plasma bound by the anti-LP-B immunosorber was negative for the presence of IgG, IgA, IgM and albumin when tested by double-diffusion analyses.

Another approach to testing the non-specific binding is to fractionate a lipoprotein sample on several immunosorbers and to examine the retained and unretained fractions by PAGE (Fig. 2). For example, small aliquots of albumin-free lipoproteins with $S_f > 12$ isolated from the plasma of a fasting normolipidemic male were applied to anti-ApoA-I, anti-LP-B, anti-ApoC, anti-ApoD and anti-ApoE immunosorbers. Care was taken not to overload each immunosorber by prior determination of the capacity of each column. The unretained and retained fractions eluted from each immunosorber were collected, placed in dialysis tubing, concentrated with polyethylene glycol, dialyzed exhaustively against the ammonium carbonate buffer and analyzed. As shown in Fig. 2,

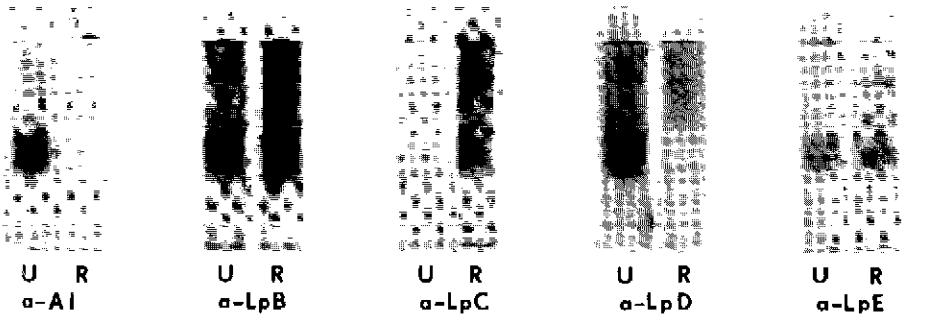

Fig. 2. Basic polyacrylamide gel electrophoresis patterns of unretained (U) and retained (R) fractions of VLDL + LDL_1 ($S_f > 12$) fractionated on different immunosorbers. Retained fractions were eluted with 3 *M* sodium thiocyanate.

there was no detectable ApoC-II or ApoC-III in the a-A-IR or a-DR fractions indicating that at least these two immunosorbers do not adsorb $S_f > 12$ lipoproteins in a non-specific fashion while the other immunosorbers fractionated $S_f > 12$ lipoproteins into unretained and retained lipoproteins.

Recovery and reproducibility

The recovery of apolipoproteins or their corresponding lipoproteins was determined by passing three aliquots of the whole plasma (WP) over two immunosorbers constructed with affinity-purified antibodies to apolipoprotein A-I and LP-B. The total recovery of four different apolipoproteins was determined: the sum of apolipoproteins recovered in the unretained and retained fractions was compared with the starting plasma levels. Good recoveries (80—98%) were obtained for all apolipoproteins tested with both anti-LP-B and anti-ApoA-I immunosorbers. A lower recovery of ApoB on the anti-LP-B immunosorber was most probably due to losses incurred during the concentration of retained fractions as illustrated in Table IV. The apolipoprotein composition of the retained fractions for each of the immunosorbers is shown in Table V. Results show that apolipoproteins other than ApoB are retained by immunosorbers specific for ApoB. The retention of small amounts of ApoC-III and ApoE is in agreement with previous results from our laboratory indicating that portions of these apolipoproteins are components of the protein moiety

TABLE V

APOLIPOPROTEIN COMPOSITION OF THE FRACTION OF WHOLE PLASMA RETAINED ON ANTI-LP-B IMMUNOSORBER

Three aliquots of two different plasma samples (WP_1 and WP_2) were chromatographed over the anti-LP-B immunosorber and the retained fraction was analyzed for apolipoproteins.

Fraction	Apolipoprotein composition (mean ± S D., n = 3) (%)			
	ApoA-I	ApoB	ApoC-III	ApoE
WP_1	5 2 ± 2.5	84.5 ± 3.7	4.4 ± 0.3	5.8 ± 3.3
WP_2	3.5 ± 2.8	89.0 ± 5 2	N.D.*	7.3 ± 2.4

*N.D. = Not detected.

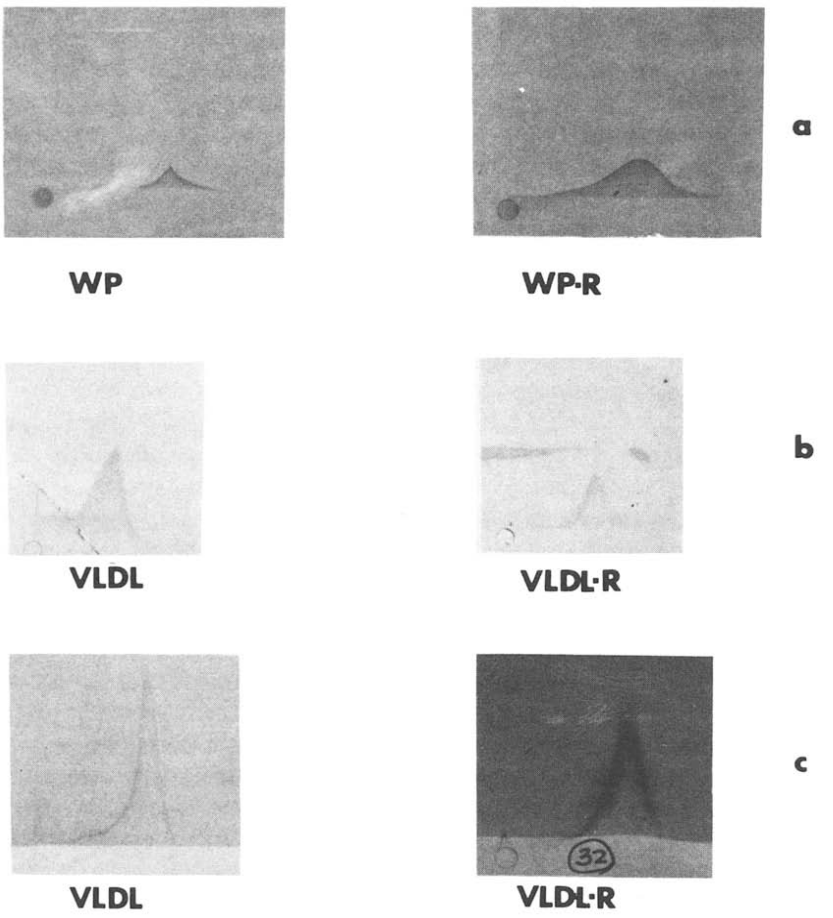

Fig. 3. Crossed immunoelectrophoresis of whole plasma (WP and VLDL) and fractions retained by anti-LP-B immunosorbers (WP-R, VLDL-R). Samples were electrophoresed in first dimension in 1% agarose and in the second dimension into gels containing antibodies to LP-B (a and b) or ApoC-III (c).

of complex lipoproteins containing ApoB [5]. To examine the structural integrity of these lipoprotein particles we compared the retained fractions from both immunosorbers with the parent whole plasma by crossed immunoelectrophoresis (Fig. 3). The mobilities of the retained fraction (WP-R) were very similar to that of parent whole plasma. These electrophoretic analyses suggest that ApoB-containing lipoproteins retained by and dissociated from Sepharose-based immunosorbers maintain their structural integrity in a fashion similar to lipoproteins isolated by other procedures. In addition, another chromatography of the retained fraction over the same immunosorber yielded no detectable apolipoproteins in the unretained fraction suggesting that the desorption of lipoproteins from immunosorbers by sodium thiocyanate does not cause dissociation of apolipoproteins from the retained ApoB-containing lipoproteins.

The reproducibility of the neutral lipid composition of lipoproteins retained by an anti-LP-B immunosorber was studied by running five aliquots of a plasma sample over the same immunosorber. There was some variability in the total

TABLE VI

NEUTRAL LIPID COMPOSITION OF FRACTIONS RETAINED BY ANTI-LP-B IMMUNOSORBERS, STUDIES ON REPRODUCIBILITY

Separate aliquots from the same plasma sample were applied five times successively to an anti-LP-B immunosorber. Immunosorber was eluted with 3 *M* sodium thiocyanate. BR_2 and BR_3 are the two retained fractions with BR_3 fraction containing a portion of the sodium thiocyanate.

Fraction	Neutral lipid* composition (%)									Total (mg per 100 ml)
	C	C-16	C-18	C-20	TG-50	TG-52	TG-54	C+CE	TG	
BR_2										
I	19.1	12.0	47.6	6.7	2.6	11.2	0.9	85.4	14.6	100.5
II	20.9	11.7	48.6	6.8	1.9	8.1	1.9	88.0	12.0	122.3
III	20.5	11.6	46.5	6.9	2.3	11.8	0.4	85.5	14.5	126.7
IV	20.5	11.9	46.5	6.8	2.4	11.4	0 5	85.7	14.3	134.6
V	20.9	12 0	47.2	6.9	2.4	9.2	1.5	87.0	13.0	161.8
Mean	20.4	11 8	47.3	6.8	2.3	10.3	1.0	86.3	13.7	129.2
S.D	0.7	0.2	0.9	0.1	0.2	1.6	0.7	1.2	1.1	22.2
BR_3										
I	21.0	11.6	47.4	7.4	3.1	8.9	0.6	87.4	12.6	13.8
II	20.5	12.0	48.2	7.5	2 6	8.5	0.7	88.2	11.8	7.6
III	21.0	10.8	46.2	7.2	2.6	11.1	1.2	85.0	14.9	21.3
IV	21.2	12.8	47.7	7.2	2.8	7.9	0.4	88.9	11.1	14.2
V	22.4	11.8	48.0	7.0	2.5	7.8	0.6	89.1	10.9	9.5
Mean	21.2	11 8	47.5	7.3	2.7	8.9	0.7	87.7	12.3	13.3
S.D.	0.7	0.7	0.8	0.2	0.2	1.3	0.3	1.6	1.6	5.3
Sum of BR_2 + BR_3										
Mean	20.5	11.8	47.3	6.9	2.4	10.1	1.0	86.5	13.5	142.4
S.D.	0.7	0.2	0.8	0.1	0.2	1.5	0.5	1.2	1.2	19.2

*Abbreviations for molecular species of lipids determined by gas chromatography are as follows: C = cholesterol; TG = triglyceride; CE = cholesterol ester; arabic numbers indicate the total acyl-carbon number of esterified fatty acids

recovered mass of neutral lipids, but the percentage composition of cholesterol esters and triglycerides (Table VI) was very similar for each retained aliquot indicating that the lipid composition of retained lipoproteins remains unchanged after repeated use of an immunosorber.

Retention of VLDL and LDL_2 by anti-LP-B immunosorbers

VLDL ($S_f > 20$) of a normolipidemic subject were isolated by the standard ultracentrifugal procedure and chromatographed on an anti-LP-B immunosorber. The retained lipoproteins were characterized by double-diffusion analysis, crossed immunoelectrophoresis (Fig. 3), and by determination of apolipoprotein (Table VII) and neutral lipid composition (Table VIII). The ApoB-containing VLDL particles were isolated both by chromatography on a Sepharose immunosorber and by immunoprecipitation in solution with the isolated IgG fraction of anti-LP-B serum. The percent composition of apo-

TABLE VII

APOLIPOPROTEIN COMPOSITION OF VLDL AND LDL_2 FRACTIONS RETAINED ON AN ANTI-LP-B IMMUNOSORBER

Fraction	Apolipoprotein composition (%)			
	ApoB	ApoC-II	ApoC-III	ApoE
VLDL*	62 5	7.4	19.3	10.7
VLDL**	61.2	4.4	19.8	13.8
VLDL-R***	67.6	6.0	15.5	11.0
VLDL-R§	67.3	7.7	14.3	10.8
LDL_2	98.0 (0.6)§§	0.2 (0.2)	0.7 (0.1)	1.0 (0.4)
LDL_2-R***	96.7 (1.3)	0.3 (0.3)	2.3 (1.5)	0.7 (1.0)
LDL_2-R§	98 1 (0.2)	0.3 (0.1)	0.2 (0.2)	1.4 (0.3)

*Parent VLDL.
**Diluted and concentrated VLDL.
***Retained fraction eluted from anti-LP-B immunosorber by sodium thiocyanate.
§Composition of fraction precipitated by addition of anti-LP-B (IgG) to isolated VLDL or LDL_2
§§Mean (S.D.), $n = 3$.

TABLE VIII

NEUTRAL LIPID COMPOSITION OF VLDL AND LDL_2 FRACTIONS RETAINED ON ANTI-LP-B IMMUNOSORBER

Fraction	n*	Lipid composition (mean ± S.D.) (%)		
		Cholesterol ester	Triglyceride	Cholesterol
VLDL	1	13.6 ± 0.3	77.9 ± 0.1	8.5 ± 0.3
VLDL-R	1	15.6 ± 0.1	74.0 ± 0.4	10.4 ± 0.4
LDL_2	5	72 3 ± 5.7	6.4 ± 1.4	21.2 ± 5.1
LDL_2-R	3	71.1 ± 7 4	3.7 ± 1.9	25 2 ± 9.0

*Number of different individual samples. VLDL analyses were performed on three separate aliquots. VLDL and LDL_2 are the parent fractions and VLDL-R and LDL_2-R are the retained fractions.

lipoproteins in the retained fractions was very similar to that of parent VLDL except for slightly higher levels of ApoB and slightly lower levels of ApoC-III (Table VII). There was also very little difference in the percent compositon of neutral lipids between the retained fractions and the parent VLDL (Table VIII). The double-diffusion pattern of the retained fraction was very similar to that of the parent VLDL. Crossed immunoelectrophoretic analyses of the parent and retained VLDL showed similar patterns against both anti-LP-B and anti-ApoC-III sera indicating the intact nature of the retained lipoproteins (Fig. 3). The presence of ApoC-III in both the retained and unretained VLDL fractions from the anti-LP-B immunosorber was consistent with the PAGE patterns (Fig. 2).

The results of immunoaffinity chromatography of VLDL isolated from patients with hypertriglyceridemia were identical to those already presented for VLDL isolated from normolipidemic subjects. Apolipoprotein analyses of

the retained fractions showed the presence of all the principal apolipoproteins of parent VLDL. In general, the apolipoprotein composition of each retained fraction was characterized by a relative increase of ApoB and a relative decrease of ApoC-III; there was no difference in the composition of neutral lipids between the retained fractions and parent VLDL.

The LDL_2 fractions were also chromatographed over an anti-LP-B immunosorber and precipitated in solution with the isolated IgG fraction of anti-LP-B serum. The percent composition of apolipoproteins (Table VII) and neutral lipids (Table VIII) of retained and preciplitated fractions was very similar to that of the parent LDL_2 preparation.

Physical properties of retained VLDL and LDL

The retained fractions of VLDL and LDL eluted from the immunosorbers were examined by analytical ultracentrifugation and electron microscopy to determine whether the elution of the retained lipoproteins with dissociating agents altered either the characteristic flotation properties or morphology.

Analytical ultracentrifugation showed that VLDL or LDL particles retained by anti-LP-B immunosorbers maintained their characteristic densities and flotation rates. The flotation rates of retained fractions from VLDL were higher than S_f 20, those of LDL_1 had flotation coefficients within the S_f 12—20 range, and those of LDL_2 within the S_f 0—12. Representative schlieren patterns are shown in Fig. 4. The retained VLDL sample showed a less heterogeneous peak with S_f 24.8 compared to the parent VLDL with its major peak of S_f 30.6. On the contrary, the retained LDL_2 sample showed a nearly identical flotation coefficient (S_f 6.3) when compared to that of parent LDL_2 (S_f 6.6). Not shown in the figures, the retained fraction of a mixture of VLDL and LDL_1 ($S_f > 12$) after passing through anti-LP-B immunosorber yielded peaks with S_f 46.2 and S_f 12.6 characteristic of $S_f > 12$ lipoproteins. These results demonstrate that the isolation of ApoB-containing lipoproteins by affinity chromatography on immunosorbers and subsequent elution with the reagents employed did not alter the physicochemical properties of the lipoprotein particles.

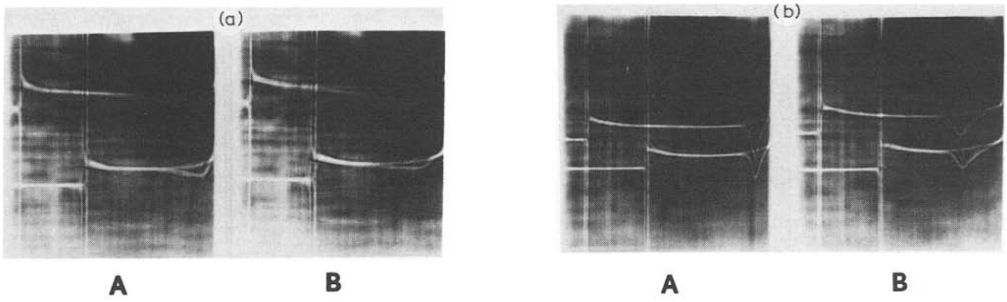

Fig. 4. (a) Schlieren patterns of VLDL and VLDL retained (VLDL-R) by an anti-LP-B immunosorber. Parent VLDL in wedge cell (top) and VLDL-R in a plain cell (bottom) at solvent density 1.063 g/ml at 20° C; pictures were taken 2 min (A) and 8 min (B) after a speed of 34 000 rpm was reached. (b) Schlieren patterns of LDL_2 and LDL_2 retained (LDL_2-R) by an anti-LP-B immunosorber. LDL_2 in a wedge cell (top) and LDL_2-R in a plain cell (bottom) at solvent density 1.063 g/ml at 20° C; pictures were taken 12 min (A) and 32 min (B) after a speed of 52 000 rpm was reached. LDL_2 was isolated from a normolipidemic subject.

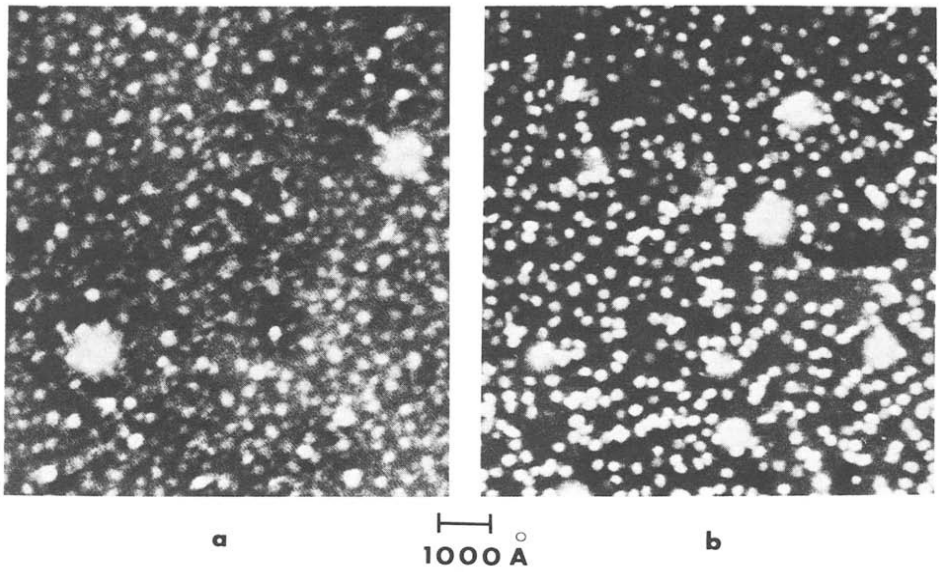

Fig. 5. Electron microscopy (negative staining, 2% phosphotungstate) of normolipidemic whole plasma (a) and the fraction of whole plasma retained (b) by an anti-LP-B immunosorber. Bar marker represents 1000 Å

Electron microscopy of the population of particles from normal plasma retained and desorbed with sodium thiocyanate from the anti-LP-B immunosorber appeared homogeneous in size (Fig. 5b). The majority of the particles were round, regularly shaped particles with a size range of LDL (220—240 Å). The other group consisted of particles > 250 Å in diameter, most of which were spherical. This group corresponds to VLDL and LDL_1 particles. The particles present in the retained fraction did not differ significantly from those present in the original plasma (Fig. 5a). This experiment showed that the anti-LP-B immunosorber retains a spectrum of ApoB-containing particles from normal fasting plasma ranging in size from LDL_2 to VLDL. From these studies, it appears that the process of binding to the LP-B immunosorber and of desorption with sodium thiocyanate does not lead to a significant change in the morphological appearance of the particles. Although all particle populations seen in the parent VLDL and LDL fractions could also be identified in the retained fractions, one cannot exclude the possibility that a selective enrichment of one or more of the particle populations may have occurred on the immunosorber. In order to address that question, comparative morphometric studies of retained and unretained fractions would have to be performed.

The electron microscopic studies in conjunction with the flotational analyses indicate that the ApoB-containing lipoproteins from normal and hyperlipidemic plasma retained by immunosorbers maintain their physicochemical properties.

Isolation of LP-B from LDL_2

Although the experiments described in the previous sections were concerned mainly with the lipoproteins retained by immunosorbers specific for ApoB, it should be pointed out that immunosorbers can also be used for fractionation

and purification of lipoproteins by removing minor simple and complex lipoprotein species through serially connected immunosorbers. This approach is especially suitable for isolating the major simple lipoprotein, LP-B, or the major complex lipoprotein, LP-A (LP-A-I:A-II).

To isolate LP-B, the LDL_2 subclass was passed sequentially over anti-ApoA-I, anti-ApoC, anti-ApoD and anti-ApoE immunosorbers. Using this approach, it was estimated that LP-B accounts for approximately 70—80% of the LDL_2 particles from normolipidemic subjects. In contrast to the parent LDL_2, the isolated LP-B (unretained fraction from the sequence of immunosorbers) only reacted with anti-ApoB (Fig. 6) and showed no bands on basic or acidic PAGE gels indicating that it was free of other known apolipoproteins. The chemical composition of the isolated LP-B (Table IX) was similar to that of ultracentrifugally isolated LDL_2 both with respect to lipid and protein content. Further studies on the characterization of LP-B preparations by determination of the phosphatide composition (Table X), fatty acid compositon of cholesterol esters and triglycerides (Table XI) and flotation coefficient were consistent with LDL_2-like lipoproteins. Studies on the biological activity of several isolated LP-B preparations with cultured fibroblasts indicated that, like LDL_2, these lipoproteins were recognized by high-affinity binding sites and

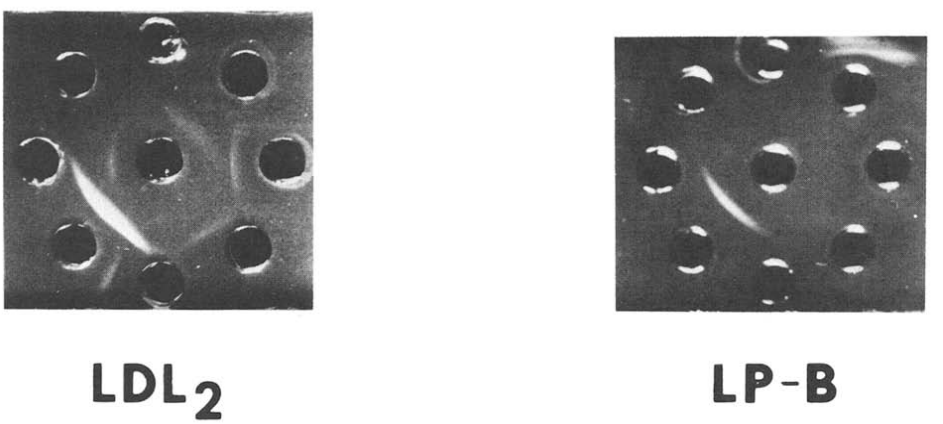

Fig. 6. Immunodiffusion pattern of LDL_2 and LP-B. Antisera placed clockwise and beginning with the top well are antisera to apolipoproteins A-II, A-I, C-III, C-II, C-I, LP-B, E, and D.

TABLE IX

CHEMICAL COMPOSITION OF LP-B ISOLATED BY IMMUNOAFFINITY CHROMATOGRAPHY

Compound	Percentage weight composition (mean + S.D., n = 5)	
	Parent LDL_2	LP-B
Triglyceride	5.25 + 0.99	9.99 ± 4.12
Cholesterol ester	41.59 ± 1.01	38.64 ± 3.93
Cholesterol	8.81 ± 0.39	7.02 + 1.23
Phospholipid	21.73 ± 1.08	20.39 ± 1.94
Protein	22.60 + 1.13	23.95 ± 4.54

TABLE X

PHOSPHATIDE COMPOSITION OF LP-B ISOLATED BY IMMUNOAFFINITY CHROMATOGRAPHY

Phosphatide	Percentage (mean ± S.D., n = 5)
Lysolecithin	5.36 ± 0.69
Sphingomyelin	27.39 ± 5.62
Lecithin	59.99 ± 8.48
Phosphatidyl serine	5.06 ± 3.42
Phosphatidyl ethanolamine	2.28 ± 1.08

TABLE XI

FATTY ACID COMPOSITION OF CHOLESTEROL ESTERS AND TRIGLYCERIDE FROM LP-B ISOLATED BY IMMUNOAFFINITY CHROMATOGRAPHY

Fatty acid	Percentage (mean ± S.D., n = 3)	
	Cholesterol esters	Triglycerides
$C_{14:0}$	0.93 ± 0.08	2.55 ± 1.03
$C_{16:0}$	19.82 ± 4.92	31.72 ± 3.60
$C_{16:1}$	3.89 ± 0.39	5.66 ± 1.52
$C_{18:0}$	1.82 ± 1.11	4.13 ± 0.41
$C_{18:1}$	25.82 ± 7.31	51.48 ± 3.61
$C_{18:2}$	42.42 ± 13.71	4.08 ± 5.83

regulated the β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase activity [46]. Based on these analyses, LP-B isolated by use of immunosorbers connected in sequence, retained its structural and biological integrity.

DISCUSSION

In this report we have presented data on the preparation of immunosorbers, isolation of the ApoB-containing lipoproteins of plasma or major density classes by immunoaffinity chromatography, and some physical and chemical properties of the isolated lipoprotein fractions.

Studies on the construction and operation of immunosorbers have led to the following conclusions. (1) Sepharose is the most suitable matrix for the column procedure. (2) Sepharose immunosorbers of highest capacity are obained by cyanogen bromide activation. (3) A comparative study on the dissociating agents showed that 3 *M* sodium thiocyanate is the most effective desorbent for lipoproteins bound to Sepharose immunosorbers; Sepharose immunosorbers treated with sodium thiocyanate retain their binding capacity to a greater degree with repeated usage than immunosorbers desorbed with buffers at low or high pH. (4) The binding capacity of immunosorbers depends not only on the selection of matrices and activation procedure but also on the selection and choice of monospecific antibodies; such antibodies can include the IgG fractions from polyclonal antisera, affinity-purified polyclonal antibodies and monoclonal antibodies; the latter two preparations of antibodies

yield immunosorbers of higher capacity because they do not contain non-immune proteins. (5) The antigen binding capacity of an immunosorber is defined as the amount of plasma apolipoprotein retained per milliliter of immunosorber per single passage; this capacity varied in the case of anti-LP-B immunosorber between 0.056 and 0.86 mg ApoB per ml of gel. (6) The possibililty of non-specific adsorption was tested by the application of human albumin (25 mg per aliquot) to immunosorbers specific for ApoA-I, ApoB, ApoC, ApoD and ApoE; of the total protein applied, 98.1% (range 96.6—100% for five columns) was present in the unretained fractions and only 2% (range 0—3.4%) in the retained fractions indicating a minimal non-specific binding of human albumin; by use of another procedure in which small aliquots of VLDL + LDL_1 were applied to immunosorbers specific for ApoA-I, ApoB, ApoC, ApoD and ApoE, it was shown that anti-ApoA-I and anti-ApoD immunosorbers do not adsorb these lipoproteins in a non-specific manner. (7) The recovery of apolipoproteins or their corresponding lipoproteins was tested with an anti-LP-B immunosorber, recoveries ranged between 80% and 98% for all apolipoproteins with the exception of ApoB; the lower recovery of ApoB on an anti-LP-B immunosorber was possibly caused by damage to the antigenic determinants of ApoB due to its instability [45]. (8) A study on the reproducibility of neutral lipid compositon of plasma lipoproteins retained by an anti-LP-B immunosorber showed that lipid composition remains unchanged after five repeated recyclings of the immunosorber.

We and others [14—20] have shown that the isolation of lipoprotein particles defined by their apolipoproteins can be accomplished by immunoaffinity chromatography. Plasma or lipoprotein density classes were chromatographed on an individual immunosorber specific for a known apolipoprotein. Retained fractions consist of lipoprotein particles containing the apolipoprotein corresponding to the specific antibodies coupled to the immunosorber. This procedure is especially suitable for the isolation of minor simple and complex lipoproteins. Alternatively, lipoproteins can be chromatographed sequentially on preselected immunosorbers with antibodies to those apolipoproteins which are not desired in the final unretained lipoprotein fraction. It should also be pointed out that, in a number of applications, the purification of a particular lipoprotein species only requires a single passage of a lipoprotein fraction over the immunosorber with the desired lipoprotein of a specific apolipoprotein composition appearing in the unretained fraction. This approach is equivalent to gel filtration of lipoproteins, a commonly used fractionation procedure considered to have little, if any, effect on the structural integrity of lipoprotein particles [37].

The effect of immunoaffinity chromatography on lipoprotein structure was tested by the isolation of Apo-B-containing particles from VLDL and LDL on anti-LP-B immunosorbers. The characterization of lipoprotein particles in retained fractions by determination of lipid and protein composition, flotation rates, double diffusion, crossed immunoelectrophoresis and electron microscopy showed that they had the chemical, physical and immunological properties of their corresponding parent density classes. These studies confirm and extend results of our previous studies and those of other investigators demonstrating the specificity of affinity chromatography as applied to fractionation and isolation of plasma lipoproteins.

We suggest that immunoaffinity chromatography in combination with micro-analytical procedures for the quantification of lipids and apolipoproteins offers an extremely powerful tool for the isolation and characterization of lipoprotein particles defined by the presence or absence of an apolipoprotein. In a broader sense, further development and application of this methodology may bring about a new phase in lipoprotein research with an emphasis on individual lipoprotein particles distinguished by their apolipoprotein composition and content. Such approaches should provide new insights into the role of apolipoproteins as the carriers of specific functions in the transport of lipids.

ACKNOWLEDGEMENTS

The authors wish to thank C. Knight-Gibson, T. Gross, J. Fesmire, R. Whitmer, J. Keeling and J. Wen for skillful technical assistance and J. Pilcher for preparation of the mansucript. These studies were supported in part by NIH Grant HL-23181.

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