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Characterization of Plasma Lipoproteins Separated and Purified by Agarose-Column Chromatography

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1. A simple method for isolation of individual human plasma lipoprotein classes is presented. In this technique, lipoproteins are removed from plasma at *d*1.225 by ultracentrifugation, after which they are separated and purified by agarose-column chromatography. 2. Three major classes are obtained after agarose-column chromatography. Separation between classes is excellent; more than 95% of the lipoproteins eluted from the column are recovered in the form of a purified lipoprotein class. 3. Each lipoprotein class was characterized immunologically, chemically, electrophoretically and by electron microscopy. A comparison of the properties of the column-isolated lipoproteins was made with very-low-density lipoproteins, low-density lipoproteins, and high-density lipoproteins separated by sequential ultracentrifugation at densities of 1.006, 1.063 and 1.21 respectively. 4. By each criterion, peak-I lipoproteins from the agarose column are the same as very-low-density lipoproteins, peak-II lipoproteins are the same as low-density lipoproteins, and peak-III lipoproteins are the same as high-density lipoproteins. Thus the lipoprotein classes isolated by both methods are similar if not identical. 5. The agarose-column separation technique offers the advantage of a two- to three-fold saving in time. In addition, the column-elution pattern serves as a recording of the size distribution of lipoproteins in plasma. 6. The most complete characterization is reported for human plasma lipoproteins. The results with rhesus-monkey and rabbit lipoproteins were identical.

Plasma-lipoprotein fractions are classically defined according to their densities (Gofman *et al.*, 1949) and may be isolated by flotation in the preparative ultracentrifuge (Havel *et al.*, 1955). Three classes of lipoproteins are separated by this technique, namely VLD lipoproteins§ (and chylomicra, when present), LD lipoproteins and HD lipoproteins. Many structural studies of lipoproteins isolated by this technique provide the basis for our current understanding of these macromolecules [see Scanu & Wisdom (1972) for review]. As the study of lipoproteins has progressed, however, many complexities have been discovered which indicate that there is heterogeneity within each class (Oncley, 1963; Levy & Fredrickson, 1965; Zilversmit, 1969; Lee & Alaupovic, 1970). The preparation of lipoproteins by sequential ultracentrifugation may cause some structural alterations, perhaps due in part to the long periods of exposure to high salt concentrations and high *g* forces involved. The presence of very-high-density lipoproteins (Alaupovic *et al.*, 1966; Scanu & Granda, 1966*a*; Albers & Aladjem, 1971) has been suggested to be due to alteration of HD lipoproteins during repeated ultracentrifugation, as has the presence of subclasses of HD lipoproteins (Levy & Fredrickson, 1965). Herbert *et al.* (1973) have described selective modification of human plasma VLD lipoproteins during repeated ultracentrifugal washing. It is difficult to determine whether the observed heterogeneity represents biological variation, modification by the preparative technique, or both. Detailed comparisons of lipoproteins isolated by classical ultracentrifugation techniques with those isolated by other methods are not available.

An alternative method of lipoprotein isolation uses agarose-column chromatography. Agarose-gel chromatography columns have been used in several laboratories to characterize circulating lipoproteins (Werner, 1966; Margolis, 1967; Kalab & Martin, 1968; Sata *et al.*, 1970, 1972; Quarfordt *et al.*, 1972). In most of these cases, individual lipoprotein classes previously isolated by preparative ultracentrifugation have been shown to be eluted as discrete fractions from agarose-chromatography columns (Margolis, 1967; Sata *et al.*, 1970, 1972; Quarfordt *et al.*, 1972).

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§ Abbreviations: VLD lipoproteins, very-low-density lipoproteins; LD lipoproteins, low-density lipoproteins; HD lipoproteins, high-density lipoproteins.

Margolis (1967) determined the molecular weights for ultracentrifugally isolated human VLD lipoproteins, LD lipoproteins and HD lipoproteins, his calculation being based on their elution volumes from calibrated agarose-chromatography columns. Thus agarose-column chromatography has been used to characterize further previously isolated plasma-lipoprotein fractions. Attempts to use it to separate VLD lipoproteins, LD lipoproteins and HD lipoproteins have been more limited.

Sata *et al.* (1970) studied the separation of human plasma lipoprotein classes after applying whole plasma to Bio-Gel A-50m [2% (w/v) agarose] chromatography columns. Although some separation was observed by these workers, a quantitative separation was not obtained, and their conclusion was that their agarose column seemed to fractionate chylomicra and VLD lipoproteins more effectively than other lipoprotein classes. Kalab & Martin (1968) obtained what appeared to be a more distinct separation of pig serum VLD lipoproteins, LD lipoproteins and HD lipoproteins. In their study a total serum-lipoprotein fraction, which had been isolated after ultracentrifugation of serum increased to a density of 1.200g/ml, was applied to a 2% (w/v) agarose column. Although some separation was clearly apparent, quantification of the separation was not given and chemical characterization of separated fractions was not presented.

In our method, we have isolated a total lipoprotein fraction by ultracentrifugation of plasma at *d* 1.225. Using Bio-Gel A-5m [6% (w/v) agarose] chromatography columns, we have preparatively separated total plasma lipoproteins into three distinct fractions, i.e. VLD lipoproteins, LD lipoproteins and HD lipoproteins, all of which are free of cross contamination. The chemical properties of each lipoprotein fraction eluted from the agarose column were found to be similar to, if not identical with, those of the respective lipoprotein fraction isolated by classical preparative ultracentrifugation techniques. The agarose-column method seems to provide an alternative preparative technique to sequential ultracentrifugation with which to evaluate the biological heterogeneity within lipoprotein classes.

A preliminary report of this work was presented at the 45th Annual American Heart Association Meeting in Dallas, Texas, U.S.A., November, 1972.

Materials and Methods

All studies were carried out on individual, unpooled samples obtained from healthy male and female subjects which were starved for 12–16h before blood collection. Humans, rhesus monkeys and rabbits were studied. Blood (20–60ml) was collected and mixed with EDTA (final concentration 2.6mM) and 5,5′-dithiobis-(2-nitrobenzoic acid) (final concentration 0.1 μM). The isolation of plasma was begun immediately after blood collection by centrifugation at 1000*g* at 15°C for 15min. The solvent density of plasma was then raised to *d*1.225 by adding solid KBr (0.3517g of KBr/ml of plasma). Plasma (*d*1.225; 10ml or less) was then placed in SW40 ultracentrifuge tubes and overlayered with *d*1.225 solution, which was prepared by addition of solid KBr to the buffered *d*1.006 solution of Scanu & Granda (1966*b*). Tubes were centrifuged in an SW40 swinging-bucket rotor for 24h at 15°C and 40000rev./min (200000$g_{av.}$) in a Beckman L2-65B ultracentrifuge. The use of a centrifuge with a rotor stabilizer greatly improved the results. The centrifuge was stopped with the brake off, tubes were carefully removed from the rotor, and the top 1.5ml containing the lipoprotein concentrate was quantitatively removed by slicing tubes with a tube slicer and then rinsing several times with small volumes of *d*1.006 solution. When necessary to apply a smaller volume to the agarose column, the final lipoprotein solution was concentrated to the desired volume by using Centriflo CF50A filter cones (Amicon Corp., Lexington, Mass., U.S.A.). This lipoprotein concentrate was found consistently to contain greater than 90% of plasma-lipoprotein cholesterol in monkeys and rabbits. In human samples, an average of 86% of the lipoprotein cholesterol was recovered in this fraction. Some of the HD lipoproteins were not present in the top layer. A second centrifugation at *d*1.225 for 24h was necessary to float the remaining HD lipoproteins. When the time of the initial centrifugation was increased to 40h, there was a 92–95% recovery of whole-plasma cholesterol in the lipoprotein concentrate from human plasma.

The lipoprotein concentrate was promptly applied, without further manipulation, to an agarose chromatography column. Columns were prepared by using either Bio-Gel A-5m, 200–400 mesh [6% (w/v) agarose content] or Bio-Gel A-15m, 200–400 mesh [4% (w/v) agarose content] (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) and were used at 4°C. Both agarose preparations gave acceptable separations. The separation between peak I and peak II (see Fig. 1) is greater on the A-15m column; the separation between peak II and peak III is greater on the A-5m column. Most of the results reported here were obtained with A-5m columns. Both 1.5cm and 2.5cm internal diam. columns were used with a bed height of 90cm. Up to 75mg of lipoprotein cholesterol was applied in a 2–4ml volume to the smaller column, which was eluted at about 10ml/h. Up to 200mg of lipoprotein cholesterol was applied in a 4–8ml volume to the 2.5cm column, and the column was eluted at about 15ml/h. The column was eluted with either 0.1M-NaCl–0.2M-potassium phosphate–0.01% EDTA, pH7.4 (Margolis, 1967), or 0.15M-NaCl–0.01% EDTA, pH7.0. No differences

were detected between lipoprotein separations with either solution; thus the latter solution was most commonly used. The eluate was monitored as E_{280}; turbidity was present in peak-I lipoproteins and increased the observed absorbance. Fractions were collected with an LKB fraction collector. The fractions of the column eluate containing individual lipoprotein peaks were combined and concentrated with filter cones to about 5–10-fold the original plasma concentration. Fractions were selected to exclude any overlap of material in adjacent peaks, although very little overlap was observed (less than 5% of lipoprotein cholesterol eluted from the column was present in the excluded fractions). Those eluted in the presence of phosphate were then dialysed for 24–48h against 6 litres of 0.15M-NaCl containing 0.01% EDTA before analysis was begun. Recovery of lipoprotein cholesterol applied to the column was usually 90% or greater. For purposes of comparison, lipoprotein fractions from some plasma samples were separated by sequential centrifugation at solvent densities of 1.006, 1.063 and 1.21 by using an SW40 swinging-bucket rotor, and were washed by re-centrifugation.

Antibodies to peak-II and peak-III lipoproteins were prepared. Lipoprotein solutions containing about 2mg of protein/ml were mixed 1:1 (v/v) with Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) and emulsified with a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y., U.S.A.). Monkey and human lipoprotein antibodies were prepared in rabbits by injection of this emulsion into foot pads. The equivalent of 2mg of protein was administered on two different dates, 10 days apart, and blood was collected 2 weeks after the second injection. Rabbit lipoprotein antibodies were prepared by subcutaneous injection of the emulsion into guinea pigs. A series of three injections each containing 1 mg of rabbit lipoprotein protein were administered 1 week apart, and the guinea pigs were bled 1 month after the first injection. Antiserum to whole human serum and antiserum to human albumin, both of which were prepared in rabbits, were purchased from Hyland, Costa Mesa, Calif., U.S.A.

Agarose electrophoresis of lipoprotein fractions was performed by a procedure modified from that of Noble (1968). The agarose concentration was 0.5% in 0.05M-sodium barbital buffer, pH 8.6, with no agar or albumin added to the agarose solution. After desalting and drying, strips were stained for 2–4h in 60% (v/v) ethanol saturated with Oil Red O and Fat Red 7B, which was maintained at 40°C. In some cases, strips were then stained with Light Green SF Yellowish, to check for plasma-protein contamination. Immunoelectrophoresis (Scheidegger, 1955) and immunodiffusion (Ouchterlony, 1958) were carried out in a 1% (w/v) agarose–0.05M-sodium barbital medium adjusted to pH 8.6.

Phospholipid phosphorus, proteins and triglycerides were determined directly on the concentrated lipoprotein solutions from the column by the methods of Fiske & SubbaRow (1925), Lowry *et al.* (1951) and Chernick (1967) respectively. Total, unesterified and esterified cholesterol were determined by *o*-phthalaldehyde as described by Rudel & Morris (1973).

Lipids were removed from each lipoprotein fraction with gentle stirring with 50vol. of ethanol–diethyl ether, 3:1 (v/v), at −10°C for 16h. The ratio of ethanol–diethyl ether was then altered to 3:5 (v/v) by the addition of diethyl ether to recover ethanol-soluble apolipoproteins, and the solution was stirred for another 6h at −10°C. The apolipoprotein precipitate was then isolated by centrifugation, resuspended in 50ml of diethyl ether and stirred at −10°C overnight. The other details of this method are as described by Scanu & Edelstein (1971). The apolipoprotein pellet was solubilized in 0.01M-Tris containing 8M-urea and applied to 7.5% (w/v) polyacrylamide gels, pH 8.9, prepared by the method of Davis (1964) and containing 8M-urea. Gels were run at 3mA/gel for 90min at 4°C. After electrophoresis, gels were fixed in 7% (v/v) acetic acid and stained with Amido Black or fixed in 10% (w/v) trichloroacetic acid and stained in Coomasie Blue, by the method of Chrambach *et al.* (1967).

Results

The elution patterns of typical separations of plasma lipoproteins isolated from young male rabbits, rhesus monkeys and humans are shown in Fig. 1. A separation into three classes of lipoproteins was obtained for each species, although the distribution of plasma lipoproteins is not the same for each. The column separation is on the basis of size. The largest lipoprotein fraction is eluted first; thus peaks I, II and III would be expected to contain VLD lipoproteins (and chylomicra, when present), LD lipoproteins and HD lipoproteins respectively. Peak IV contains 5,5′-dithiobis-(2-nitrobenzoic acid), which is a low-molecular-weight compound (mol. wt. 396) and serves as a marker for the total volume of the gel bed. For the 90cm column of 1.5cm internal diam., the total volume is 180ml, and the V_e/V_t (V_e is the elution volume and V_t is the total volume of the gel bed) values (±S.D.) for peaks I, II and III were 0.38±0.009, 0.560±0.009 and 0.730±0.009 respectively in both human and rabbit samples. The E_{280} for each lipoprotein peak, though not directly proportional to concentration, is an estimate of the amount of material present; thus elution patterns describe the size distribution of lipoproteins of individual plasma samples.

Agarose electrophoresis was used initially to aid the characterization of the lipoproteins eluted from the column. Plate 1(*a*) shows the results obtained

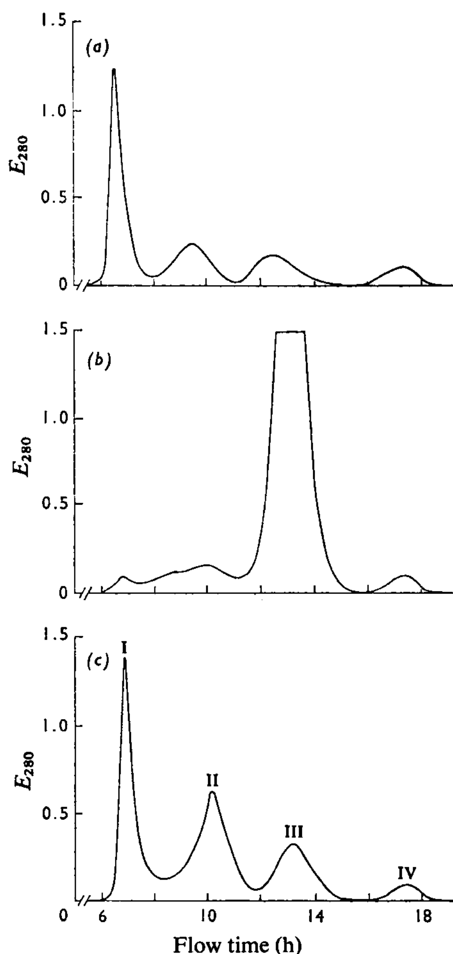

Fig. 1. *Separation of plasma lipoprotein classes by agarose-column chromatography*

Elution patterns of plasma lipoproteins isolated from a representative male rabbit (*a*), rhesus monkey (*b*) and human (*c*). Lipoproteins were quantitatively removed from plasma by ultracentrifugation of plasma at *d*1.225 and were then applied to a Bio-Gel A-5m agarose column, 1.5cm×90cm, and eluted in 0.15M-NaCl–0.01% EDTA at 4°C.

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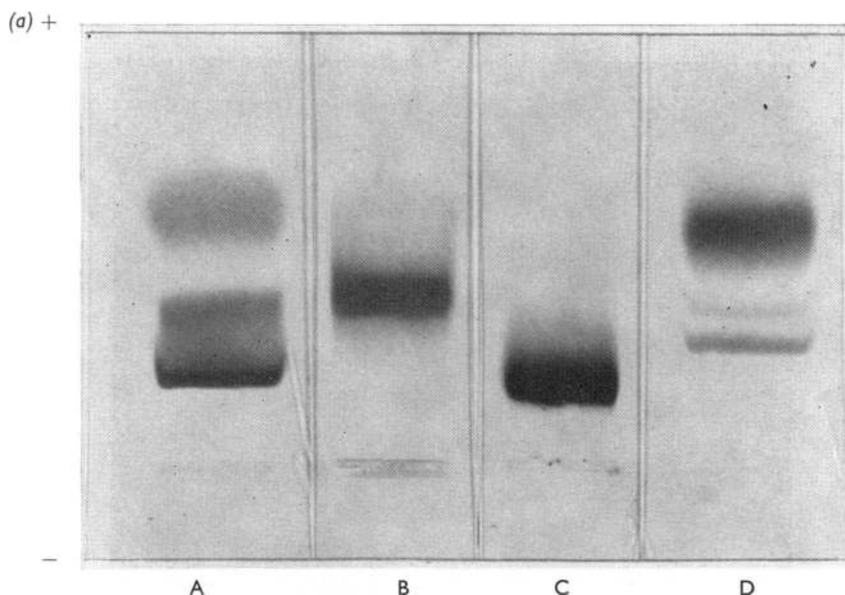
after the column fractions from a human plasma sample were concentrated in Amicon filter cones. Peak-I lipoproteins have primarily pre-β-mobility, which is characteristic of VLD lipoproteins. Peak-II lipoproteins have β-mobility which is characteristic of LD lipoproteins. Peak-III lipoproteins have primarily α-mobility, characteristic of HD lipoproteins. After concentration, additional bands in the pre-β region were consistently found in peak-III lipoproteins as minor bands, although the amount of material found in the pre-β region varied among individuals. The sample of peak-III lipoproteins shown in Plate 1(*a*) contained the most pre-β material that we have observed. In many samples, only one pre-β band was apparent in the peak-III concentrate.

Immunoelectrophoresis of column-prepared lipoproteins was performed as a routine. Plate 2 shows the results obtained for each peak when compared with antisera prepared to peak-II and peak-III lipoproteins. The peak-I lipoproteins reacted with both anti-(peak II) and anti-(peak III) antisera, whereas peak-II and peak-III lipoproteins reacted only with their respective antibody preparations. The peak-I lipoproteins showed a precipitin line which appeared to be discontinuous at the top (see drawing in Plate 2). When each lipoprotein peak was tested immunoelectrophoretically against anti-(whole serum), no additional lines developed and no reaction with anti-albumin occurred for any of the column peaks. Each precipitin line shown in Plate 2 was shown to stain for lipid and protein. It has been reported that plasma VLD lipoproteins isolated ultracentrifugally do not react with antisera to HD lipoproteins until partially depleted of lipid (Levy *et al.*, 1966; Gustafson *et al.*, 1966). Therefore we tested VLD lipoproteins isolated by ultracentrifugation with our anti-(peak III) antiserum and found the same result as for peak-I lipoproteins.

Immunodiffusion was also performed (Plate 1*b*). Peak-I lipoproteins cross reacted with anti-(peak II) and anti-(peak III) antisera, whereas peak-II lipoproteins and peak-III lipoproteins cross reacted only with their respective antisera. The double band found for peak III versus anti-(peak III) antiserum was also present in whole plasma versus anti-(peak III) antiserum. VLD lipoproteins, LD lipoproteins and HD lipoproteins prepared in the ultracentrifuge showed similar immunoreactivity as peak-I, peak-II and peak-III lipoproteins respectively.

Each column-prepared lipoprotein class was examined by using electron-microscope techniques (Jones & Price, 1968). The results shown in Plate 3 demonstrate heterogeneity in the size of peak-I lipoproteins, with the particles found to be between 25 and 90nm (250–900Å). Such a size distribution is also characteristic of human VLD lipoproteins isolated by ultracentrifugation (J. Quan, A. L. Jones & R. L. Hamilton, personal communication). The peak-II material is homogeneous in size and has an approximate diameter of 20nm (200Å), which is also characteristic of ultracentrifugally isolated LD lipoproteins. Peak-III lipoproteins are 6–12.5nm (60–125Å) in size, which is also characteristic of ultracentrifugally isolated HD lipoproteins, although a few particles greater than 25nm (250Å) in size were seen in this fraction.

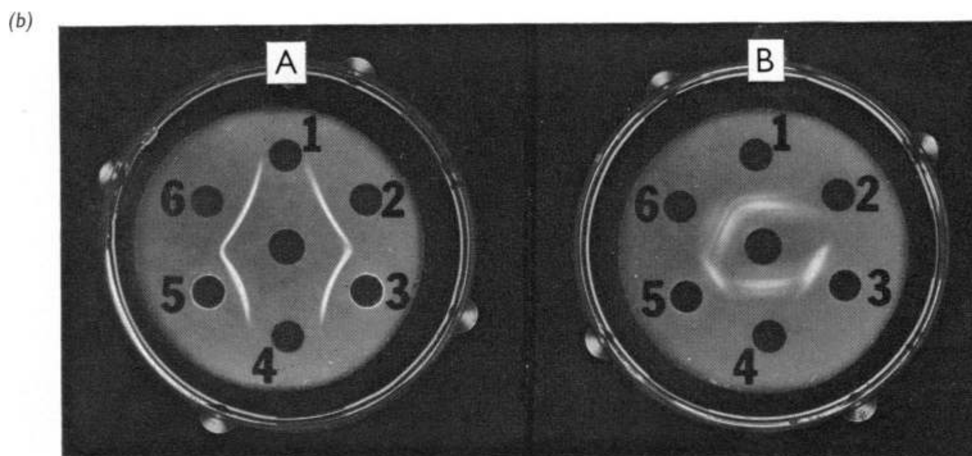
The chemical composition of column-prepared lipoprotein classes was compared with that of



EXPLANATION OF PLATE 1(a)

Agarose-electrophoresis patterns of human lipoprotein classes separated by agarose-column chromatography

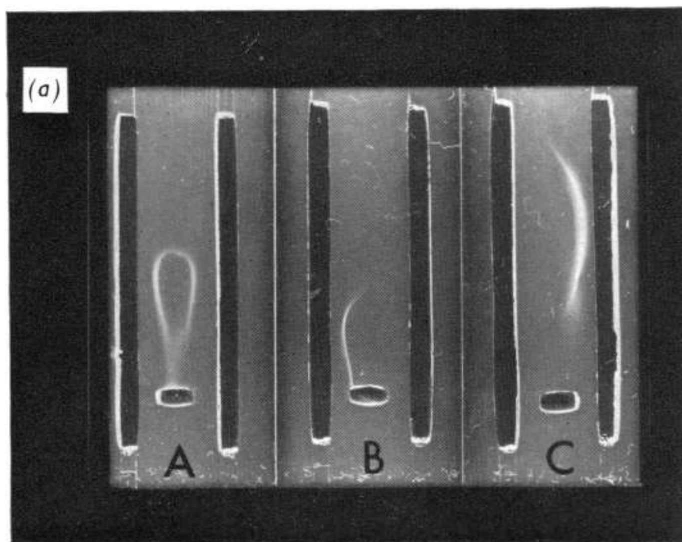
Human whole-plasma sample (A) collected 14h after a meal, and the peak-I (B), peak-II (C) and peak-III (D) lipoproteins isolated from it. Whole plasma (15μl) and lipoprotein fractions (15μl each) were placed in the wells. The material in each peak from the column (see Fig. 1) was combined and concentrated to approx. ten times the original plasma concentration.

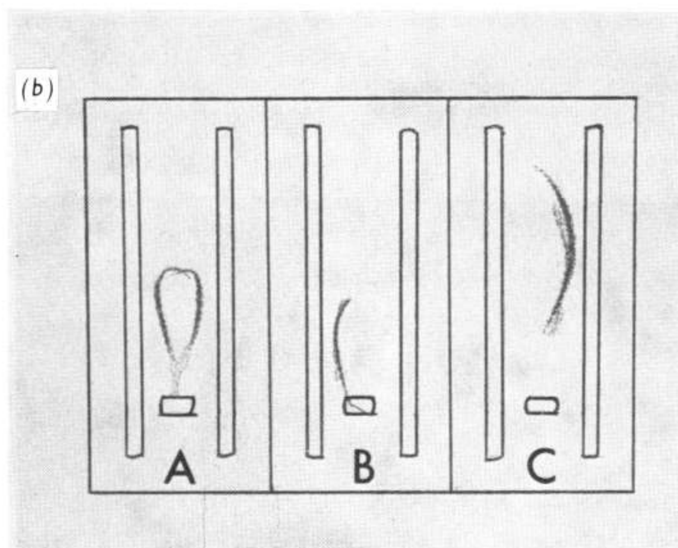


EXPLANATION OF PLATE 1(b)

Immunodiffusion of human lipoproteins separated by agarose-column chromatography

Centre well of disc A contained 20μl of rabbit anti-(human peak-II lipoprotein) antiserum and centre well of disc B contained 20μl of anti-(human peak-III lipoprotein) antiserum. For both discs, the outside wells numbered in clockwise fashion contained: (1) peak-III lipoprotein; (2) peak-II lipoprotein; (3) peak-I lipoprotein; (4) peak-III lipoprotein; (5) peak-I lipoprotein; (6) whole plasma. The equivalent of 30μg of lipoprotein protein was placed in each well and 5μl of whole plasma was used. Diffusion was complete at 96h and photographs were taken.





EXPLANATION OF PLATE 2

Immunoelectrophoresis of human lipoproteins separated by agarose-column chromatography

The left trough of each slide contained rabbit antiserum to human peak-II lipoproteins and the right trough of each contained rabbit antiserum to human peak-III lipoproteins. To start the run, peak-I lipoproteins containing 10 μg of protein were placed in well A, peak-II lipoproteins containing 12 μg of protein were placed in well B and peak-III lipoproteins containing 20 μg of protein were placed in well C. Slides were run at 6mA/slide for 70min, after which electrophoresis was stopped and 50 μl of antisera was placed in the troughs. Diffusion was complete at 48h and photographs were taken as shown in (*a*). (*b*) is a line drawing of the slides.

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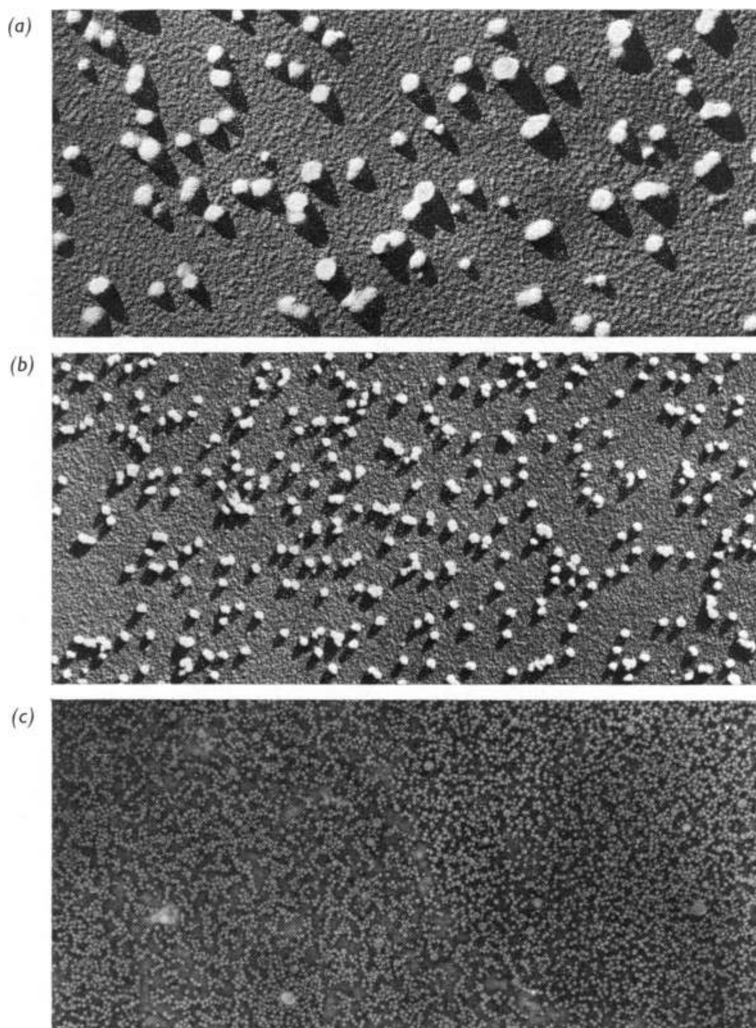
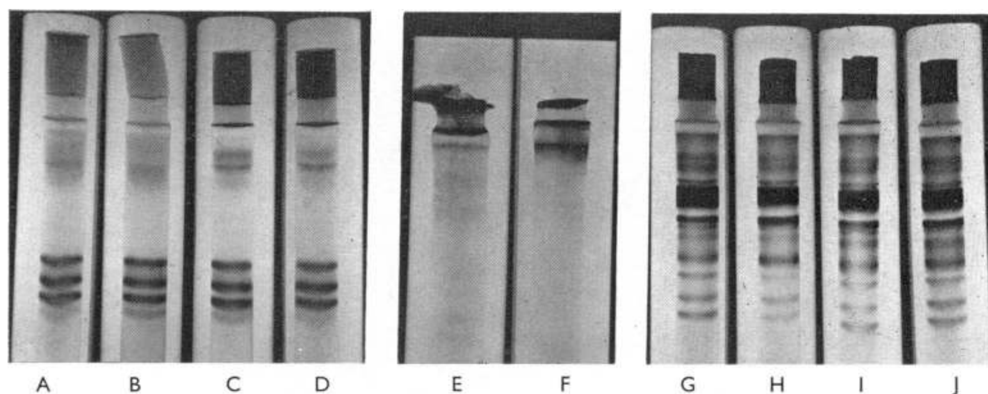


EXPLANATION OF PLATE 3

Electron micrographs of the three major human-lipoprotein fractions isolated by agarose-column chromatography

Magnification is about ×46000. (*a*) shows peak-I lipoproteins fixed in OsO_4 and shadowed with platinum. (*b*) shows peak-II lipoproteins processed as above. Note the uniform diameter of this exceptionally pure fraction of LD-lipoprotein-sized particles. (*c*) shows peak-III lipoproteins negatively stained with 1% (w/v) phosphotungstic acid. For particles less than 15nm (150Å) in size negative staining is the superior technique, since it does not require the additional metal coating.



EXPLANATION OF PLATE 4

Polyacrylamide-gel patterns of apolipoproteins of agarose column and ultracentrifugally isolated human lipoproteins

Each gel contained the resolubilized apolipoproteins from the following delipidated lipoprotein preparations: A and B, peak-I lipoproteins; C and D, VLD lipoproteins; E, peak-II lipoproteins; F, LD lipoproteins; G and H, peak-III lipoproteins; I and J, HD lipoproteins. Between 40 and 60μg of protein was applied to each gel and the gels were stained with Coomasie Blue.

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Table 1. *Chemical composition of human lipoproteins isolated by agarose-column chromatography and by sequential ultracentrifugation*

Individual plasma samples were divided in half for lipoprotein isolation by both methods as described in the Materials and Methods section. Each value represents the mean±S.E.M. for determinations performed in duplicate on lipoprotein preparations from three individuals.

	Percentage of total weight					Ratios	
Lipoprotein fraction	Free cholesterol	Cholesteryl ester	Phospholipid	Triglyceride	Protein	Total cholesterol / Phospholipid	Esterified / Total cholesterol
Peak I	6.1 ±0.7	12.8 ±3.3	19.6 ±1.2	47.7 ±6.2	13.5 ±2.0	0.71	0.56
VLD lipoproteins	5.4 ±0.7	12.6 ±0.2	21.5 ±1.8	41.8 ±3.7	11.6 ±0.7	0.62	0.58
Peak II	9.4 ±0.2	39.9 ±1.2	22.6 ±0.3	5.4 ±0.3	21.6 ±0.3	1.50	0.72
LD lipoproteins	8.8 ±0.1	42.1 ±1.4	23.7 ±0.1	5.1 ±1.7	19.9 ±0.7	1.44	0.74
Peak III	3.2 ±0.2	20.8 ±0.7	25.2 ±0.6	7.7 ±1.9	43.7 ±1.5	0.64	0.79
HD lipoproteins	3.0 ±0.4	21.4 ±1.1	29.0 ±0.9	2.1 ±2.1	43.8 ±3.9	0.55	0.81

ultracentrifugally isolated VLD lipoproteins, LD lipoproteins and HD lipoproteins (Table 1). The compositions of the lipoproteins prepared by column chromatography are similar to, if not identical with, those isolated by sequential ultracentrifugation.

Plate 4 shows a comparison between apolipoprotein patterns of column-prepared and ultracentrifugally isolated lipoprotein fractions. The patterns are similar for peak-I lipoproteins and VLD lipoproteins, and for peak-III lipoproteins and HD lipoproteins. Most of the apolipoproteins failed to enter the gel for both peak-II lipoproteins and LD lipoproteins.

Discussion

A method using agarose-column chromatography is described, in which preparative separation of all major plasma lipoprotein classes is obtained and documented. Several points about this technique are essential to the success of the method and differentiate it from other attempts to use agarose-column chromatography for preparative separation of lipoproteins (Kalab & Martin, 1968; Sata *et al.*, 1970). (1) Total plasma lipoproteins are quantitatively removed from plasma by a single centrifugation in a manner in which plasma-protein contamination of the total lipoprotein mixture is avoided. (2) The separation between lipoprotein classes by using the present method was good. Isolated VLD lipoproteins, LD lipoproteins and HD lipoproteins were free from contamination by other lipoprotein classes, and free from contamination by plasma proteins. Less than 5% of the total eluted lipoprotein cholesterol was excluded when major classes were prepared by combining the fractions under each elution peak. (3) Each fraction eluted from the column has been characterized chemically, immunologically, electrophoretically and by electron microscopy, and has been shown to be chemically similar to its equivalent fraction isolated by sequential ultracentrifugation.

To expand on each of these three points, the following comments seem pertinent. Point 1: we have monitored the completeness of separation of total lipoproteins from plasma by determining the percentage of cholesterol recovered in the top fraction after ultracentrifugation compared with the amount in the plasma placed in the ultracentrifugation tube. Whereas VLD lipoproteins and LD lipoproteins are quantitatively recovered there are differences between samples in the percentage of HD lipoproteins that reach the top of the tube after a 24h centrifugation. We could increase the recovery of HD lipoprotein in the total lipoprotein fraction by increasing the time of centrifugation. In all cases, it was possible to set the time of centrifugation to recover greater than 95% of plasma-lipoprotein cholesterol in the total lipoprotein fraction. An important aspect of purification of lipoproteins by the method described here is in the initial ultracentrifugation step. When whole plasma is carefully overlayered with *d*1.225 solution to serve as a 'wash' layer and is then centrifuged in a swinging-bucket rotor with rotor stabilizer, the total lipoprotein concentrate at the top of the tube has been found to be free from contaminating plasma proteins, as demonstrated immunologically and by protein staining of agarose-electrophoresis strips. None of the lipoprotein fractions are packed into a sedimented

plasma-protein pellet. Instead lipoproteins are washed free from other plasma proteins in the initial centrifugation step and albumin contamination is not a problem. On the other hand, significant amounts of albumin were found in the lipoprotein concentrate when samples were isolated in the absence of a wash layer or when a rotor stabilizer was not used. The albumin was usually eluted as a small peak after peak-III lipoproteins but was incompletely separated from peak III. It was possible to purify further the albumin-contaminated peak-III material by using an 8% (w/v) agarose column (Bio-Gel A-1.5m; Bio-Rad Laboratories).

Points 2 and 3: we determined as a routine the total percentage recovery of lipoprotein from the column, as monitored by cholesterol determination. Recovery appeared to be essentially complete, with an average of 94% being recovered. We selected the three lipoprotein classes from each column separation by excluding the fractions between the peaks and only combining those fractions which were actually under individual peaks. Essentially no cross-contamination was found (Plates 1*a*, 1*b* and 2) when this was done, even when more than 95% of the eluted lipoprotein cholesterol was included in the final combination of fractions. Thus we make the statement that the separation between major lipoprotein classes is excellent and describe the method as a preparative method. In our experience, more of the lipoprotein cholesterol present in a plasma sample can be recovered by using the column technique than by using sequential centrifugations on a preparative ultracentrifuge.

An important fact is that the chemical properties of column-isolated lipoproteins agreed closely with those of lipoproteins prepared by sequential ultracentrifugation. Modifications of 'native' lipoproteins occurring during either method of preparation do not appear to cause major structural changes, unless the same changes were induced by both methods. This seems unlikely. The fact that the isolated lipoprotein products are the same recommends the column method, since it can be completed in a fraction of the time needed for sequential ultracentrifugation. Another advantage of the column technique is that the size distribution of lipoproteins in the plasma sample is recorded during separation by monitoring the column eluate at 280nm and the efficiency of separation is thereby determined. A comparison of the elution patterns of the human and rhesus-monkey lipoproteins (Fig. 1) shows that separation of individual classes is obtained in spite of a significant difference in distribution. The agarose-column technique has proved useful in detecting abnormal patterns of lipoprotein distribution.

In the present studies, immunological techniques ave been used to characterize lipoprotein preparations. Peak-I lipoproteins and ultracentrifugally isolated and washed VLD lipoproteins both react to anti-(peak-III) antiserum on immunoelectrophoresis and immunodiffusion. This would appear to be in disagreement with the results of others (Levy *et al.*, 1966; Gustafson *et al.*, 1966), but seems reasonable considering what is now known about the apolipoproteins of VLD lipoproteins and HD lipoproteins. Many workers have shown apparent identity between some of the apolipoproteins of VLD lipoproteins and HD lipoproteins (Scanu & Wisdom, 1972). Bilheimer *et al.* (1972) and Eisenberg *et al.* (1972) have demonstrated the exchange of some radioactive apolipoproteins between HD lipoproteins and VLD lipoproteins. The polyacrylamide-gel patterns in the present paper show that peak-I and peak-III lipoproteins are similar to VLD lipoproteins and HD lipoproteins respectively, and share common apolipoproteins. Since at least some of the apolipoproteins common to HD lipoproteins and VLD lipoproteins appear to exchange *in vivo*, it is reasonable to assume they are on the surface of the VLD lipoprotein molecules and are therefore antigenically available.

Our results show a slight heterogeneity in peak-III material. The evidence for this was the presence of pre-β bands on agarose electrophoresis, seen immunoelectrophoretically and by immunodiffusion as a double-line pattern, and the presence of a few 25nm (250Å) particles as seen by electron microscopy. The nature of this minor component in peak III is partially described by the properties it has displayed with these techniques, i.e. (1) it behaves on the agarose column as if it is the same size as HD lipoproteins, although larger particles are infrequently seen with electron microscopy which may represent the minor component; (2) it remains present in peak III after as many as three consecutive column purifications; (3) it migrates to the pre-β region on agarose electrophoresis, but no reaction between peak-III lipoproteins and anti-(peak II) antiserum was found. Therefore it does not have the immunological properties of typical VLD lipoproteins. It is not known if this minor lipoprotein of peak III could be similar to two additional minor HD-lipoprotein groups described by Barclay *et al.* (1963, 1965). The latter group has reported two lipoprotein peaks in the analytical ultracentrifuge which appear in addition to the major HD-lipoprotein peak in the plasma-lipoprotein fraction isolated between *d*1.063 and *d*1.125.

In summary, we have described a method by which plasma lipoproteins can be quantitatively isolated from plasma and preparatively separated into major classes by using agarose-column chromatography. The agarose-column-chromatographic separation can be completed in 1 day and offers the advantage that the size distribution of lipoproteins is monitored

and recorded while separation is proceeding. Peak-I lipoproteins and VLD lipoproteins, peak-II lipoproteins and LD lipoproteins, and peak-III lipoproteins and HD lipoproteins are chemically very similar and are probably identical. It appears that column-isolated lipoprotein classes may prove useful in evaluating biological heterogeneity within individual classes.

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