FRACTIONATION OF LIPOPROTEINS FROM BLOOD BY GEL FILTRATION*

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The wide variations in the amounts of lipoproteins circulating in blood plasma were first observed with the dark field microscope[1]. Electron microscope and ultracentrifugal studies suggest a continuous, multimodal distribution ranging from lipoproteins to large chylomicrons of approximatively 500 mμ diameter.

This paper describes fractionation of lipoproteins from blood by gel filtration. In this procedure chromatography is performed on columns of small porous particles or gel beads. Separation operates by diffusional partition of solutes between a stationary phase, within the bed material, and a mobile phase, exterior to it. Solute molecules which are small enough to penetrate the porous gel beads are retarded relative to larger molecules unable to enter the pores.

A gel with a fairly loose framework is necessary for the fractionation of lipoproteins. Among the bed materials available at present (*e.g.*, latex rubber, starch, dextran, polyacrylamide) agar offers the largest pore sizes. Agarose, a neutral constituent of agar[2], was used to avoid specific adsorption of solutes to the bed matrix, as chylomicrons are known to be easily adsorbed on charged surfaces.

EXPERIMENTAL

Preparation of agarose gel beads

Agarose was prepared from commercial agar (Baltimore Biological Laboratory) according to the method of HJERTÉN[3,4]. 1 or 2 % agarose was gelled in water by autoclaving (121°, 15 lb/in.², 15 min). The gel was left standing for 24 h at 4° to set. Beads were prepared by pressing the gel twice through a metal sieve of mesh size 30. Fines were washed off by exposing the beads to a water jet on a sieve of mesh size 60. The resulting slurry of beads (mesh size 30 to 60) was suspended in water. Air was removed by stirring, under vacuum, with a magnetic stirrer for 1 h. The beads can be stored for several months in the cold under water.

Preparation of the columns

Columns with a total bed volume of 120 ml (height about 70 cm, inner diameter 1.5 cm), and with a total volume of 200 ml (height about 40 cm, inner diameter 2.5 cm) were prepared. The aqueous suspension of granulated agarose was poured into the

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columns which consisted of glass tubes provided with a coarse sintered-glass filter at the lower end, on which glass beads (2 mm diameter) were placed to a height of 1 cm. This prevented clogging of the filter by agarose beads during prolonged use. Glass-wool cannot be used for this purpose since it adsorbs chylomicrons. No solvent flow was allowed during the packing of the column. Excess water after sedimentation of the granules was sucked off from the top.

Operation of columns

Phosphate buffer 0.2 *M*, pH 7.4, was used as eluent in all experiments. The columns were operated at room temperature. Prior to use they were washed with 500 ml buffer to elute the water of the original suspension, and soluble matter. The sample was layered on top of the agarose bed, which was never allowed to run dry, and washed in with repeated aliquots of buffer (1 to 4 ml). Buffer was subsequently supplied from a pressure head of 40 to 60 cm. This caused new columns to shrink slightly. The eluates were collected in an automatic fraction collector. Usually 4–12 h were required for the fractionation of one sample. Maximum flow rate was limited by the packing of a particular column. Handled properly, columns could be used repeatedly.

Analytical methods

Protein content was estimated spectrophotometrically at 280 mμ in a Beckman DU Spectrophotometer. Turbidity was estimated either with a locally made nephelometer in arbitrary units, or spectrophotometrically at 700 mμ. Lipids were extracted according to the method of FOLCH[5]. Total lipids were determined by the dichromate method using either BRAGDON's[6] modification for large concentrations, or that of PANDE, KHAN AND VENKITASUBRAMANIAN[7] for small concentrations. Cholesterol was estimated directly in the eluates by SEARCY, BERGQUIST AND JUNG's[8] method, with slight modifications. For thin layer chromatography of the lipid extracts mixtures of petroleum ether–ethyl ether (95:5, and 90:10, v/v) were used. Spots were rendered visible by charring with 50 % H_2SO_4, and heating at 200° for 1 h.

Test substances

The following substances were used to test the efficiency of separation, and to calibrate the columns (data are taken from recent literature):

1. Blood of *Helix pomatia* containing as the major protein hemocyanin with a molecular weight of 6,680,000 (diffusion coefficient $1.38 \cdot 10^{-7}$ cm^2/sec).
2. Purified hemocyanin from the blood of *Limulus polyphemus*, molecular weight 1,300,000.
3. Dyed dextran 2000, obtained by hydrolysis from a bacterial polysaccharide (Pharmacia, Uppsala, Sweden), average molecular weight 2,000,000.
4. Human anti-polio globulin (E. R. Squibb and Sons, New York, N.Y.), molecular weight 150,000 (diffusion coefficient $4.5 \cdot 10^{-7}$ cm^2/sec).
5. Bovine serum albumin, molecular weight 70,000 (diffusion coefficient 5.9×10^{-7} cm^2/sec).

Calculation of the distribution coefficient, K_d

The effluent volume V_e for a given particle species is given by the equation

describing the operational parameters of gel filtration[9]:

$$V_e = V_o + K_d (V_{tot} - V_o) = V_o + K_d V_i \quad (1)$$

V_{tot} is the total solvent volume of the column, V_o the volume of liquid exterior to the gel phase, V_i the volume of liquid within the gel phase. K_d, the distribution coefficient, characterizes the behavior of a given particle species within the gel. It is obtained by transforming eqn. (1) to:

$$K_d = \frac{V_e - V_o}{V_{tot} - V_o} = \frac{V_e - V_o}{V_i} \quad (2)$$

K_d is equal to zero for all particles that are excluded from the gel phase, and equal to one for all particles that diffuse unrestrictedly into the gel phase. When the substance tested does not interact with the bed material, and gel filtration is the only discriminating factor, K_d values are thus confined to the interval zero to one.

RESULTS

Column calibration

Five test substances ranging from a molecular weight of 70,000 to 6,680,000 were run on a column of 2 % agarose with a 200 ml total bed volume (Fig. 1). Blood of *Helix pomatia* produced two peaks, the first (K_d = 0.348) due to hemocyanin, the second due to contaminating proteins. Dextran 2000 also produced two peaks, the first (K_d = 1.000) due to large particles of unhydrolyzed polysaccharide, and the second (K_d = 0.591) to particles with an average molecular weight of 2,000,000. The breadth of the second peak indicated inhomogeneity of particle size. *Limulus polyphemus* blood produced a single peak (K_d = 0.609) with slight tailing, probably due to an impurity of hemocyanin. Single symmetrical peaks were obtained from human γ-globulin (K_d = 0.809), and bovine serum albumin (K_d = 0.826). In Fig. 2, K_d values of the test substances are correlated with the logarithm of their molecular weight.

Separation of particles from blood

Fig. 3 shows the elution patterns of human blood serum of a normal subject from a column of 2 % agarose with a total bed volume of 120 ml. Serum obtained in the fasting state (top) eluted slightly earlier than globulin, and albumin eluted on the larger column used for calibration in a symmetrical peak (K_d = 0.72). After a fat load consisting of 100 ml heavy cream (bottom) a first peak containing all the turbid material appeared with the front (K_d = 0), separated from the second peak.

The elution pattern of dog serum, heavily lipemic after corn oil feeding (total lipids 3750 mg %), resembled that of man (Fig. 4). A column of 2 % agarose with a total bed volume of 200 ml was used. Two separated peaks appeared, the first (K_d = 0) coinciding with the elution of turbid liquid, the second (K_d = 0.83) representing the bulk of plasma proteins. Lipid analysis also showed two peaks, the first coinciding with the front, the second (K_d = 0.73) slightly preceding the plasma protein bulk. Of the 112.5 mg lipid in the sample put on the column, 107.3 mg was recovered in the eluates.

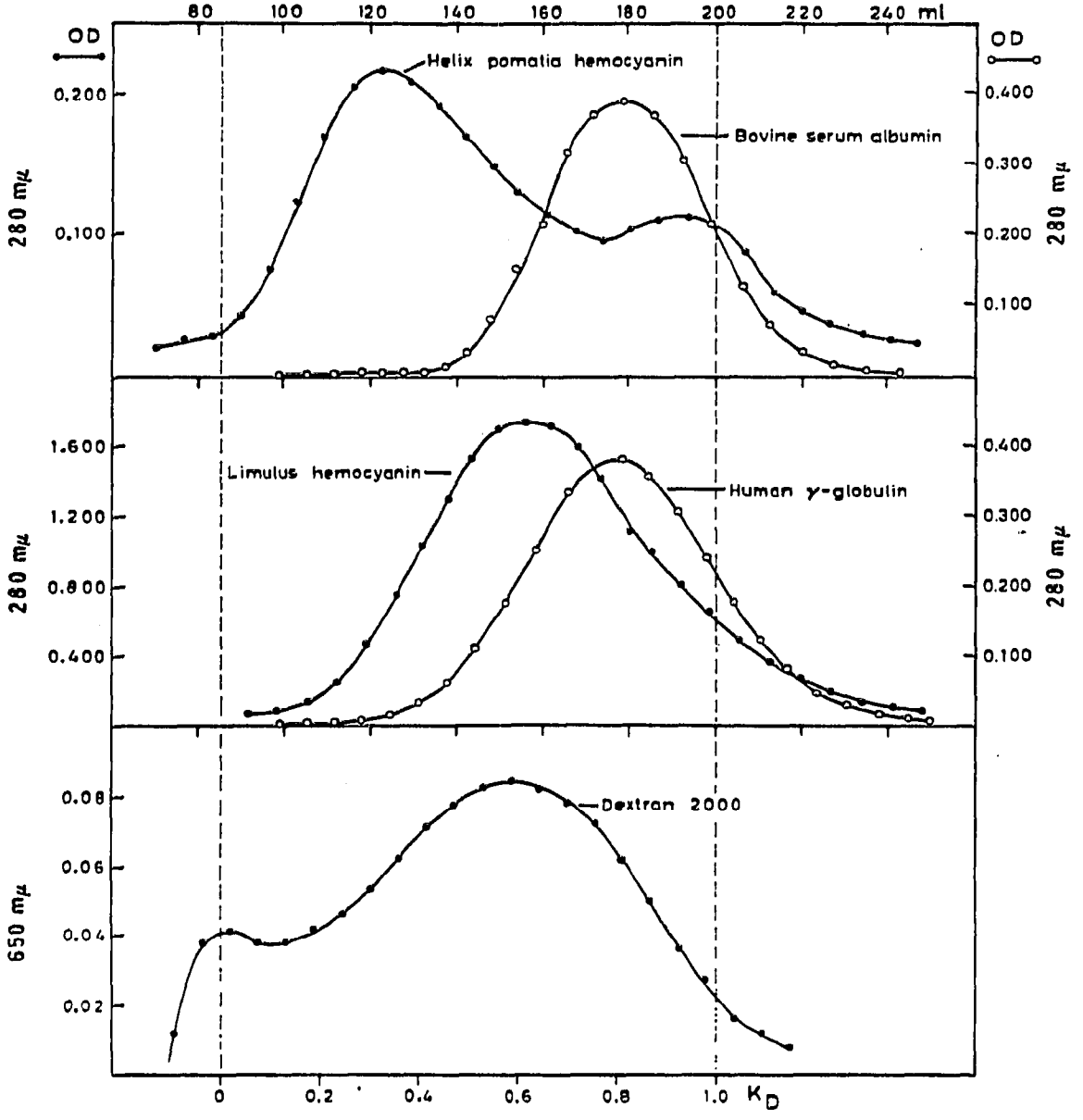

Fig. 1. Elution patterns of *Helix pomatia* hemocyanin, *Limulus polyphemus* hemocyanin, Dextran 2000, human γ-globulin, and bovine serum albumin from a column of 2 % agarose beads.

A different pattern was obtained from serum of a fasting woman with hyperlipemia (total lipids 2910 mg %), as shown in Fig. 5. A column of 2 % agarose with a total bed volume of 200 ml was used. Turbid liquid eluted with a maximum at K_d = 0.35. Measurement of optical density at 280 mμ showed a single peak (K_d = = 0.74), with a marked shoulder in the ascending limb (K_d = 0.40). Lipid analysis gave a single peak skewed to the right (K_d = 0.45). Of the 116.4 mg lipid put on the column, 115.8 mg was recovered in the eluates.

The serum of a 35 year old male with essential hyperlipemia (total lipids 2460 mg %) gave the pattern in Fig. 6. The same column as before was used. Blood was withdrawn one hour after a breakfast consisting of orange juice, and coffee. Turbid liquid eluted with the front. Measurement of the optical density at 280 mμ showed two

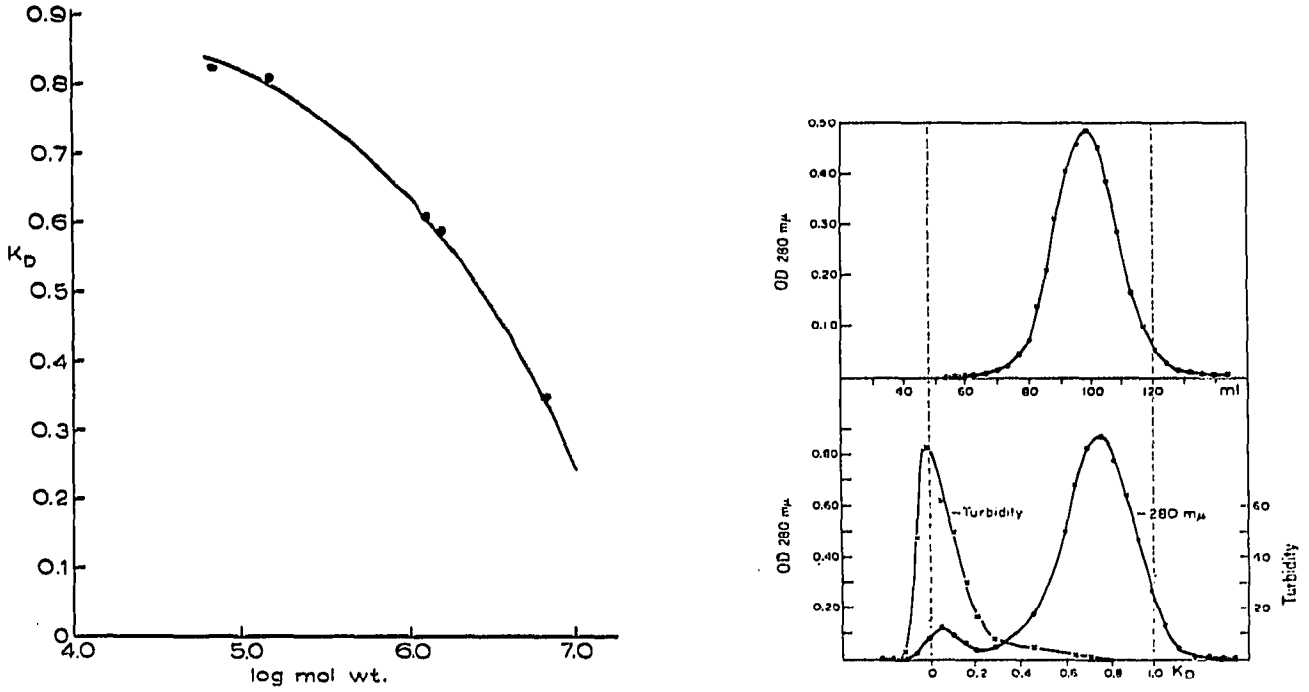

Fig. 2. Relationship between K_d values, and molecular weight. The results of the runs plotted in Fig. 1 were used.

Fig. 3. Chromatography of human blood serum on a column of 2% agarose beads. Top: 2 ml of fasting serum. Bottom: 4 ml of serum withdrawn after peroral lipid load. (Eluates were diluted 1:10 for measurement of optical density at 280 mμ).

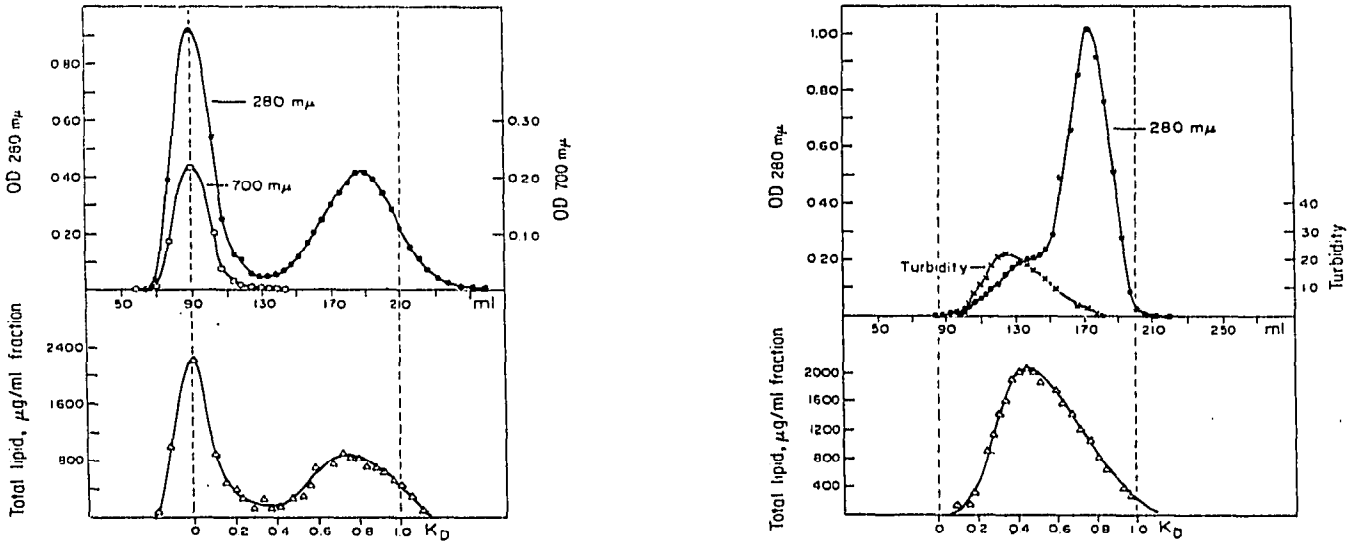

Fig. 4. Chromatography of 3 ml lipemic dog serum (total lipids 3750 mg%) on a column of 2% agarose beads. Top: optical densities at 280 mμ (eluates diluted 1:10), and 700 mμ. Bottom: total lipid content of eluates.

Fig. 5. Chromatography of 4 ml fasting serum of a woman with hyperlipemia (total lipids 2910 mg%) on a column of 2% agarose beads. Top: optical densities at 280 mμ (eluates diluted 1:10), and turbidity. Bottom: total lipid content of eluates.

peaks (K_d = 0 and K_d = 0.80) connected by a zone of overlap. Lipid analysis of the eluates showed a very broad peak with a maximum at K_d = 0.15, and a shoulder reaching to K_d = 0.70. Total cholesterol concentration increased during elution from about 15 % to 35 % of total lipids. Thin layer chromatography revealed free cholesterol which paralleled triglyceride concentration, while the concentration of

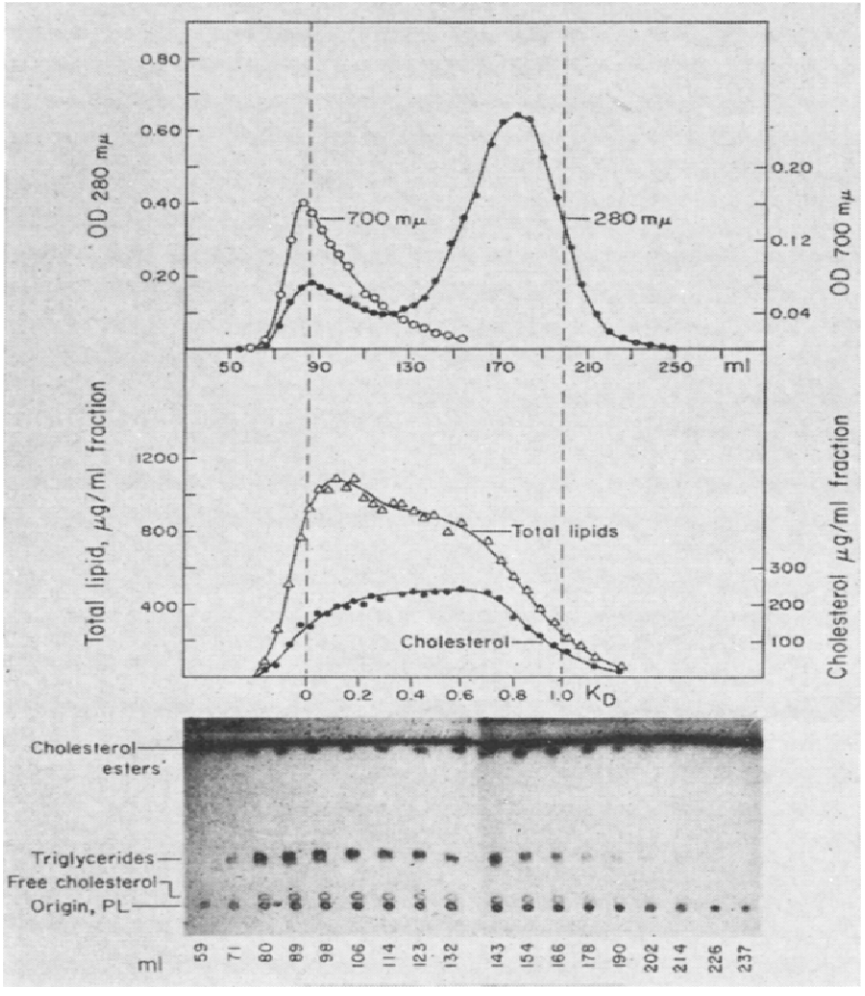

Fig. 6. Chromatography of 5 ml serum of a man with hyperlipemia (total lipids 2460 mg %) on a column of 2 % agarose beads. Top: optical densities at 280 mμ (eluates diluted 1 : 10), and 700 mμ. Middle: total lipid, and total cholesterol content of eluates. Bottom: thin layer chromatography of lipid extracts of eluates.

cholesterol esters increased later in the run. Of the 123.0 mg lipid put on the column, 108.3 mg was recovered in the eluates.

The diagrams in Fig. 7 were obtained with fasting serum from a 49 year old male with carbohydrate induced hyperlipemia (total lipids 6420 mg %). Turbid liquid eluted in a strongly skewed peak with the front. Measurement of optical density at 280 mμ showed two overlapping peaks (K_d = 0 and K_d = 0.80). Total lipid content of the eluates showed a single, strongly skewed peak with a maximum at K_d = 0.05, and a shoulder at K_d = 0.60. Total cholesterol eluted in a similar pattern. During elution cholesterol increased from 15 % to 20 % of total lipids. Thin layer chromatography showed both free, and esterified cholesterol to parallel the concentration of

triglycerides. Of the 315.0 mg % lipid put on the column, 248.8 mg was recovered in the eluates.

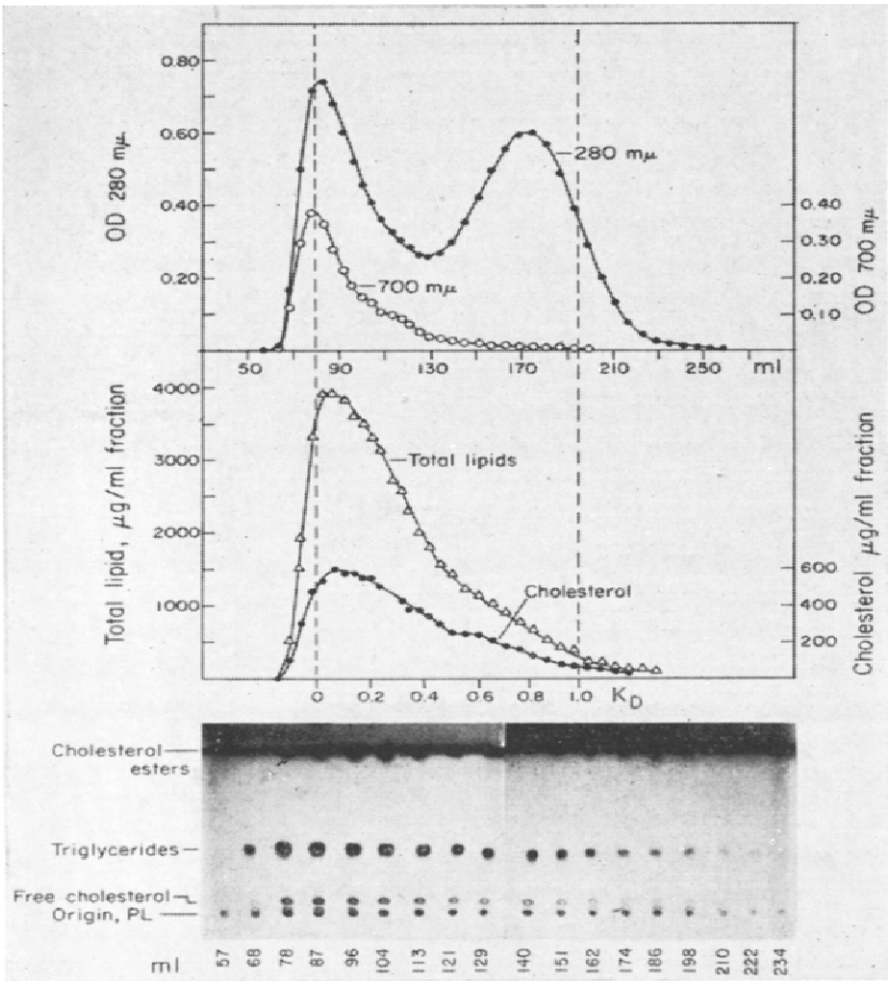

Fig. 7. Chromatography of 5 ml serum of a man with hyperlipemia (total lipids 6420 mg %) on a column of 2 % agarose beads. Top: optical densities at 280 mμ (eluates diluted 1:10), and 700 mμ. Middle: total lipid, and total cholesterol content of eluates. Bottom: thin layer chromatography of lipid extracts of eluates.

DISCUSSION

The use of agar gels as bed material has expanded the range of gel filtration to very large molecules, and to viruses[10,11]. The nature of serum lipoproteins excludes drastic or aggressive fractionation procedures. In gel filtration the sample is subjected only to the forces of diffusion, and constantly remains in buffered solution. Good reproducibility of the distribution coefficient, K_d, and of the column parameters, V_o and V_t, was found on similarly sized columns. Sometimes K_d values for the same substance however may differ on columns of varying dimensions. Recovery, as measured by analysis of total lipids, was usually over 90 %.

ACKERS[12] has calculated an average pore radius of 108 mμ for 2 % agar gel. On the basis of his formulas we arrived at a similar value of 104 mμ average pore radius using globulin as test substance (molecular radius 5.22 mμ; K_d = 0.809).

TABLE I

EXPECTED K_d VALUES OF DIFFERENT LIPOPROTEIN CLASSES ON A COLUMN OF 2% AGAROSE GEL BEADS

Lipoprotein sizes are taken from FREEMAN *et al.*[13].

Lipoprotein class	*Molecular weight*	*Expected K_d*
Alpha	150,000–400,000	0.80 – 0.74
S_f 0– 20	$1 \cdot 10^6$– $3 \cdot 10^6$	0.65 – 0.52
S_f 20–400	$5 \cdot 10^6$–$12 \cdot 10^6$	0.42 – 0.16
S_f 400– 10^5	$12 \cdot 10^6$– $3 \cdot 10^{11}$	Front – 0.16

However different values were obtained for albumin (molecular radius 3.70 mμ; K_d = 0.826; calculated average pore radius 82 mμ), and *Helix pomatia* hemocyanin (molecular radius 32 mμ; K_d = 0.348; calculated average pore radius 149 mμ).

Expected K_d values for the various lipoprotein classes were calculated on the basis of their molecular weight (Table I). They fit the experimental data. In lipemic plasma from normal humans (Fig. 3), and dogs (Fig. 4) two classes of lipoproteins were separated. Small lipoproteins (alpha-lipoproteins, and S_f 0 to 20 class) eluted around K_d = 0.75, large chylomicrons (S_f 400 to 10^5 class) with the front. In one case representative of hyperlipemia (Fig. 5) lipoproteins causing turbidity were found around K_d = 0.35 (S_f 20 to 400 class). Other patients with hyperlipemia (Figs. 6 and 7) showed a continuous distribution of lipoproteins over the whole range of separation with varying importance of the different size classes.

SUMMARY

A new method for the fractionation of lipoproteins from blood by gel filtration on 2% agarose is described. Effective separation of nearly spherical particles in the range of molecular weight 10^5 to 10^7 can be achieved. Recovery of sample in the eluates is practically complete. Representative patterns obtained from hyperlipemic blood samples are presented.

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