ESTIMATION OF BLOOD LIPOPROTEINS BY RADIAL IMMUNODIFFUSION AFTER AGAROSE GEL FILTRATION

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Gel filtration on columns of 2 % agarose gel beads is capable of separating nearly spherical particles in the molecular weight range 10^5 to 10^7 (ref. 1). This allows the fractionation of blood plasma lipoproteins: The framework of the gel is sufficiently loose to retard α_1-lipoproteins, β-lipoproteins and lipid particles up to the S_f 400 class; larger particles elute with the front. In addition to monitoring the eluates for turbidity (at 700 mμ), and protein content (at 280 mμ) it is possible to analyze their total lipid content, and its varying components. Since the lipoprotein classes differ in their composition, this will give characteristic patterns. More direct information would however be obtained by quantitation of the individual lipoproteins themselves in the eluates.

This paper describes analysis of serum lipoproteins, and other proteins, after fractionation by gel filtration by radial immunodiffusion[2]. In this method a specific antibody is incorporated into a layer of gelled agarose. Wells are punched out, and samples of the elution fractions placed in them. The antigen-containing sample then diffuses into the antibody-containing agarose, where specific antigen–antibody complexes precipitate in an extending circular area until all antigen is used up. The final area of precipitation is proportional to the antigen concentration in the sample.

EXPERIMENTAL

Preparation and operation of columns

Details have been previously described[1]. Columns with a total bed volume of 200 to 230 ml (height 40 to 45 cm, inner diameter 2.5 cm) were prepared from gelled agarose beads (mesh size 30 to 60). Phosphate buffer (0.2 *M*, pH 7.4) was used as eluent. The high molarity was chosen to exclude adsorption, and aggregation of lipoproteins. Usually 10 ml freshly drawn serum was used as sample.

Preparation of radial immunodiffusion plates

An exactly 0.1 cm thick polyethylene strip (about 0.5 cm width) was put between two glass plates (20 × 10 cm) along the two narrow ends. The plates were then made watertight by sealing with polyethylene adhesive tape (Shandon Labortechnik, Frankfurt) the two narrow edges, and one long side of the plate, thus forming

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a flat chamber. Before pouring the agarose–antibody mixture into this, the plates were warmed to 50° in an incubator.

0.25, 0.50 or 0.75 g agarose (Industrie Biologique Française) were dissolved in 20 ml Michaelis buffer (sodium diethylbarbituric acid 0.1 *M*, pH 8.6) in a water bath at 90° to 100°. The solution was cooled to about 48°, antiserum added in the desired concentration, and the volume of the mixture brought to 25 ml with buffer (final agarose concentration: 1 %, 2 %, or 3 %). Antiserum and agarose solution were mixed with a glass rod, avoiding the formation of air bubbles. The mixture was then poured immediately between the pre-warmed glass plates. Once the agarose has gelled the plates are ready for use. They can be kept at 4° for weeks.

Plasma protein antisera from the rabbit (Behringwerke, Marburg) were used in the following concentrations: anti-α_1-lipoprotein serum, 2 %; anti-β-lipoprotein serum, 1 %; anti-α_2-macroglobulin serum, 0.2 % and 0.5 %; anti-antitrypsin serum, 2 %; anti-acid-α_1-glycoprotein serum, 2 %; anti-γG-globulin serum, 4 %. The concentrations had to be adjusted to the size of the original serum sample, and to the potency of the antiserum.

Radial immunodiffusion

The adhesive tape was removed from the plates, and one glass plate detached from the agarose layer. (It is possible to siliconize one glass plate prior to use to facilitate this.) Sample wells (1.6 mm diameter) were punched out with a suction needle (as used for immunoelectrophoresis, Beckmann Instruments). Usually a distance of 12 to 15 mm from well to well was chosen. 2 μl of antigen-containing sample solution were placed in each well with a Hamilton pipette. For immunodiffusion the plates were kept in a high humidity chamber at room temperature (usually 24 h). The non-precipitated proteins were then eluted with saline for 24 h, the precipitates fixed, and stained with saturated amidoblack solution (amidoblack 10 B 25 g, methanol 1250 ml, distilled water 1000 ml, acetic acid 250 ml), and the plates decolorized with methanol–glacial acetic acid (9:1; v/v). The diameters of the precipitates were measured in two directions at right angles with a lens fitted with a scale reading to 1/10 mm, the square of the radius being used as a measure of the precipitation area. For documentation the agarose layer can be dried under a moist filter paper, and removed from the glass plate as a film.

Calibration curves were made from dilutions of a standard serum for each individual plate. The results were expressed in per cent concentration of this standard.

Other analytical methods

Optical density measurements at 280 mμ and 700 mμ were made spectrophotometrically on a Zeiss PMQ spectrophotometer. Lipids were extracted according to the method of FOLCH *et al.*[3], and total lipids determined by the dichromate method[4,5]. Alternatively the lipids were estimated directly in the eluates with the sulfophosphovanillin method[6,7] after lyophilization (100 μl samples). This method, while avoiding tedious extraction of lipids, gave the same results as the dichromate method. Cholesterol was estimated directly in the eluates by the method of SEARCY, BERGQUIST AND JUNG[8], with slight modifications. For thin-layer chromatography of the lipid extracts mixtures of petroleum ether–ethyl ether (90:10, v/v) were used. Spots were rendered visible by charring with 50 % H_2SO_4 and heating at 200° for 1 h.

RESULTS

Fig. 1 is the photograph of an immunodiffusion plate, where α_1-lipoproteins have been precipitated by a specific antiserum. Samples of successive elution fractions of human serum separated on a column of 2 % granulated agarose were placed in the different wells. Reading from the top and each row from right to left, the antigen concentration increases to a maximum, then decreases. Dilutions of a standard serum used for calibration are in the bottom row. Samples stored for a prolonged period at 4° sometimes produced a second inner ring, when α_1-lipoprotein was precipitated. It only appeared if the α_1-lipoprotein concentration was high and was always proportional to the main outer ring. No other antiserum produced double rings.

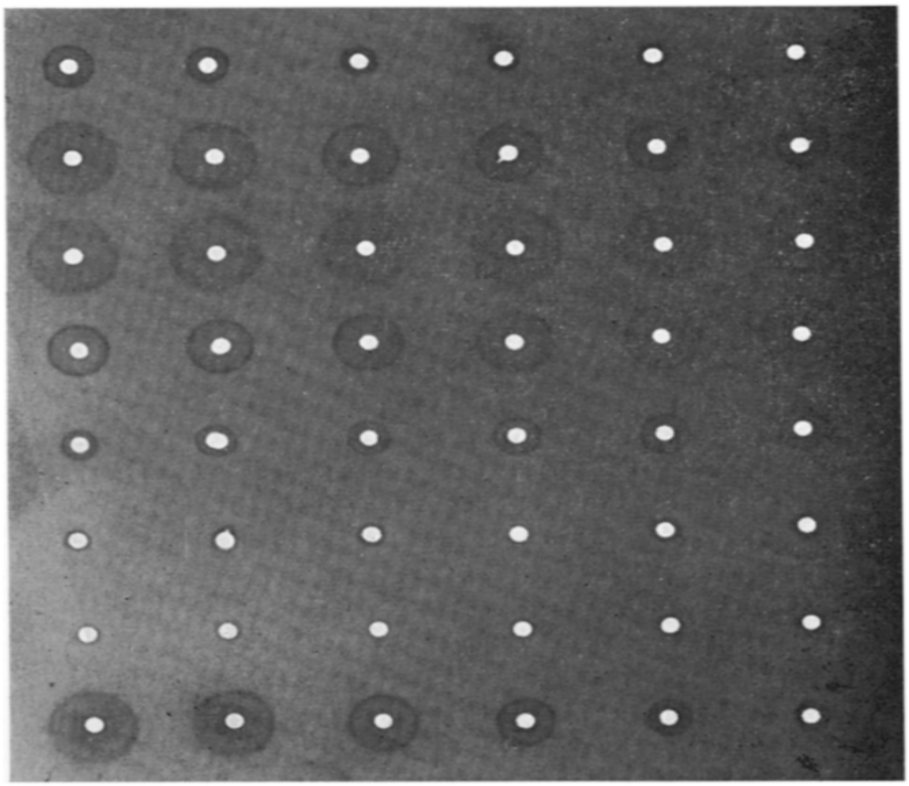

Fig. 1. Radial immunodiffusion plate: 2 % (v/v) of anti-α_1-lipoprotein was gelled in a 1 mm thick layer of 2 % agarose. Well diameter 1.6 mm. 2 μl of individual fractions of serum separated by gel filtration were placed in the wells. The bottom row are dilutions of a standard serum. Amido-black stain of the antigen–antibody precipitates.

Fig. 2 shows the pattern obtained from normal blood serum, drawn 3 h after a load of 250 ml heavy cream (total lipids 705 mg/100 ml), separated on 2 % granulated agarose. Measurement of optical density at 280 mμ showed two well separated peaks, where the first at the front (K_d 0) contained all the turbid liquid, as measured at 700 mμ. α_1-Lipoproteins eluted in a single symmetrical peak with a maximum between K_d 0.77 and K_d 0.85. β-Lipoproteins eluted earlier with a maximum between K_d 0.50 and K_d 0.62, in a skewed peak. Its shape was identical, whether 1 or 3 % agarose was used in the radial immunodiffusion. Total lipid content of the eluates

attained a maximum between the β- and α_1-peaks. Some lipid was present in the early fractions where the large chylomicrons appeared; these did not react with either the α_1- or β-antibody. Antitrypsin eluted in a symmetrical peak at K_d 0.81 to K_d 0.85. α_2-Macroglobulin eluted earlier with a maximum between K_d 0.62 and K_d 0.65 in a skewed peak, due to molecular size variations.

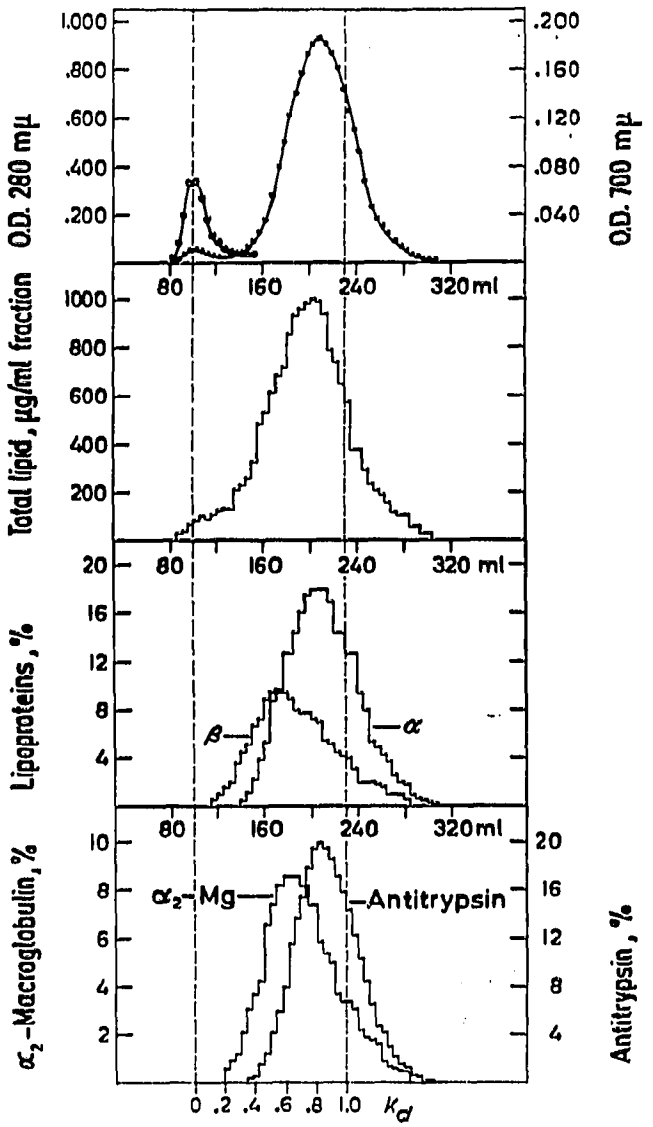

Fig. 2. Chromatography of 10 ml normal human blood serum, withdrawn 3 h after peroral lipid load (total lipids 705 mg %), on a column of 2% agarose beads. Optical densities at 280 mμ (eluates diluted 1:10), and 700 mμ. Total lipid content of eluates. α_1-Lipoprotein, β-lipoprotein, α_2-macroglobulin and antitrypsin concentrations of eluates, expressed in per cent of a standard serum.

The major lipoprotein classes and their estimated molecular weights[9] are listed in Table I, at the left. On the right are the K_d values expected from the calibration of 2% granulated agarose beds[1], and the K_d values found by immunodiffusion. Except for a slight retardation of the α_1-lipoproteins the values agree well. No appreciable interaction occurs between the bed material and the lipoproteins, which are known to be easily adsorbed on charged surfaces.

TABLE I

MOLECULAR WEIGHTS OF DIFFERENT LIPOPROTEIN CLASSES (DATA TAKEN FROM FREEMAN *et al.*[9]) EXPECTED K_d VALUES ON A COLUMN OF 2 % AGAROSE GEL BEADS (DATA TAKEN FROM WERNER[1]) AND K_d VALUES FOUND BY RADIAL IMMUNODIFFUSION

Lipoprotein class	*Molecular weight*	*K_d value*	
		Expected	*Found*
Alpha	150,000 — 400,000	0.80 — 0.74	0.85 — 0.77
S_f 0 — 20	1×10^6 — 3×10^6	0.65 — 0.52	0.62 — 0.50
S_f 20 — 400	5×10^6 — 12×10^6	0.42 — 0.16	
S_f 400 — 10^5	12×10^6 — 3×10^{11}	Front — 0.16	

A summary of K_d values of various proteins on 2 % and 4 % granulated agarose as determined by radial immunodiffusion is given in Table II. 2 % agarose is more suitable for the separation of chylomicrons (K_d 0 to K_d 0.42) from lipoproteins, but practically no resolution is obtained among proteins smaller than γG-globulin. On 4 % agarose, the β-lipoproteins shift towards the front and are better separated from the α_1-lipoproteins, but most chylomicrons will elute in bulk with the front. On the other hand separation is effective down to molecular weights of 45,000.

The diagrams in Fig. 3 were obtained with two blood samples from the same patient with hyperlipemia due to alcoholism and liver cirrhosis (total lipids 1140 mg %). The sample on the left was taken 3 h after a load of 300 ml heavy cream. Chylomicrons in large quantity eluted in a sharp peak at the front (optical density 700 mμ). Measurement at 280 mμ showed two well separated peaks. The total lipid curve also showed two peaks: The smaller, first one, eluted at the front; the larger, second one, preceded its normal K_d value slightly. K_d values for β- and α_1-lipoproteins coincided with those in normals. The sample on the right was taken 8 h after the same meal. Chylomicrons were only partially cleared, turbid liquid still eluted at the front. Although the total lipid content of the serum remained unchanged, there was less lipid at the front and comparatively more in a shoulder between K_d 0.2 and K_d 0.4; the main peak retained its position. Simultaneously the β-lipoprotein concentration increased, while that of α_1-lipoprotein remained unchanged.

Serum obtained in the fasting state from a woman with essential hyperlipemia (total lipids 3700 mg %) gave a different pattern (Fig. 4). The turbid liquid (optical density 700 mμ) eluted in a strongly skewed peak at the front. Measurement of optical

TABLE II

K_d VALUES OF VARIOUS PROTEINS DETERMINED BY RADIAL IMMUNODIFFUSION AFTER FRACTIONATION ON COLUMNS OF 2 % AND 4 % AGAROSE GEL BEADS

Protein	*Molecular weight*	*K_d on 2 % agarose*	*K_d on 4 % agarose*
β-Lipoproteins	1×10^6 — 3×10^6	0.62 — 0.50	0.35 — 0.26
α_2-Macroglobulin	820,000 — 950,000	0.65 — 0.62	0.40 — 0.35
α_1-Lipoproteins	150,000 — 400,000	0.85 — 0.77	0.65 — 0.61
γG-Globulin	150,000	0.85 — 0.81	0.65 — 0.57
Antitrypsin	45,000	0.85 — 0.81	0.70 — 0.65
Acid α_1-glycoprotein	44,000	0.88 — 0.81	0.70 — 0.65

density at 280 mμ showed a shoulder in the ascending limb, starting at the front and continuing uninterruptedly to the main peak. Total lipid eluted in a single peak, starting at the front. Its maximum at K_d 0.4 appeared earlier than in normals and in the previous case. In spite of the large quantity of chylomicrons the proteins reacting with β- and α_1-antigen retained their peak positions. β-Lipoproteins were present in larger amounts than normal. Some α_1-lipoprotein reaction was found at early K_d values, almost up to the front. Thin-layer chromatography showed slightly differing maxima for the individual lipid classes; cholesterol esters were maximal at 140 ml, triglycerides and free cholesterol at 160 ml.

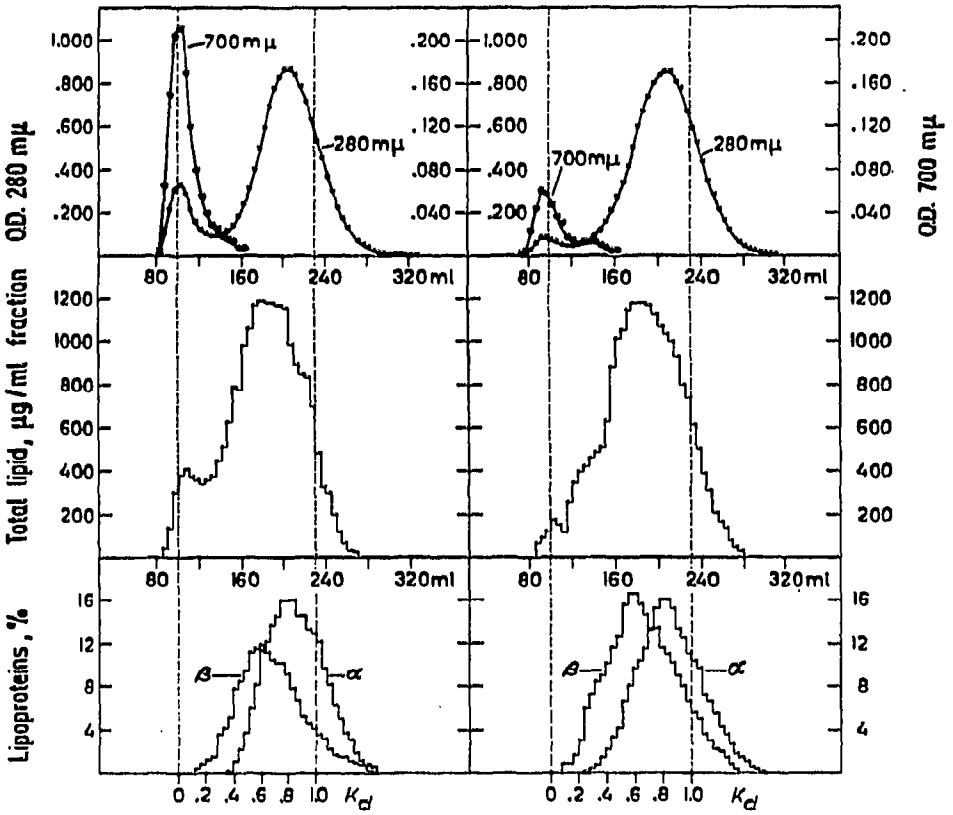

Fig. 3. Chromatography of 10 ml human blood serum of a man with hyperlipemia due to alcoholism and liver cirrhosis (total lipids 1140 mg %) on a column of 2 % agarose beads. Left: Serum withdrawn 3 h after peroral lipid load. Right: Serum withdrawn 8 h after the same meal. Optical densities at 280 mμ (eluates diluted 1:10), and 700 mμ. Total lipid content of eluates. α_1-Lipoprotein and β-lipoprotein concentrations are expressed in per cent of a standard serum.

A different pattern (Fig. 5) again was obtained from serum drawn in the fasting state from a patient with carbohydrate induced hyperlipemia and xanthomatosis (total serum lipids 3900 mg %). The marked turbidity of the serum eluted mostly at the front. Measurement of optical density at 280 mμ showed two distinct peaks. The maximum of total lipid content was shifted close to the front as lipid analysis and thin-layer chromatography showed. All major lipid classes eluted with a maximum around 110 ml (K_d 0.2).

DISCUSSION

The lipids of plasma do not circulate free, but occur in specific combinations with protein and carbohydrate[10]. Major classes of these complexes can be separated by electrophoresis[11], ultracentrifugation[9], or insoluble complex formation[12]. For the fractionation of larger particles (very low density lipoproteins, chylomicrons) special

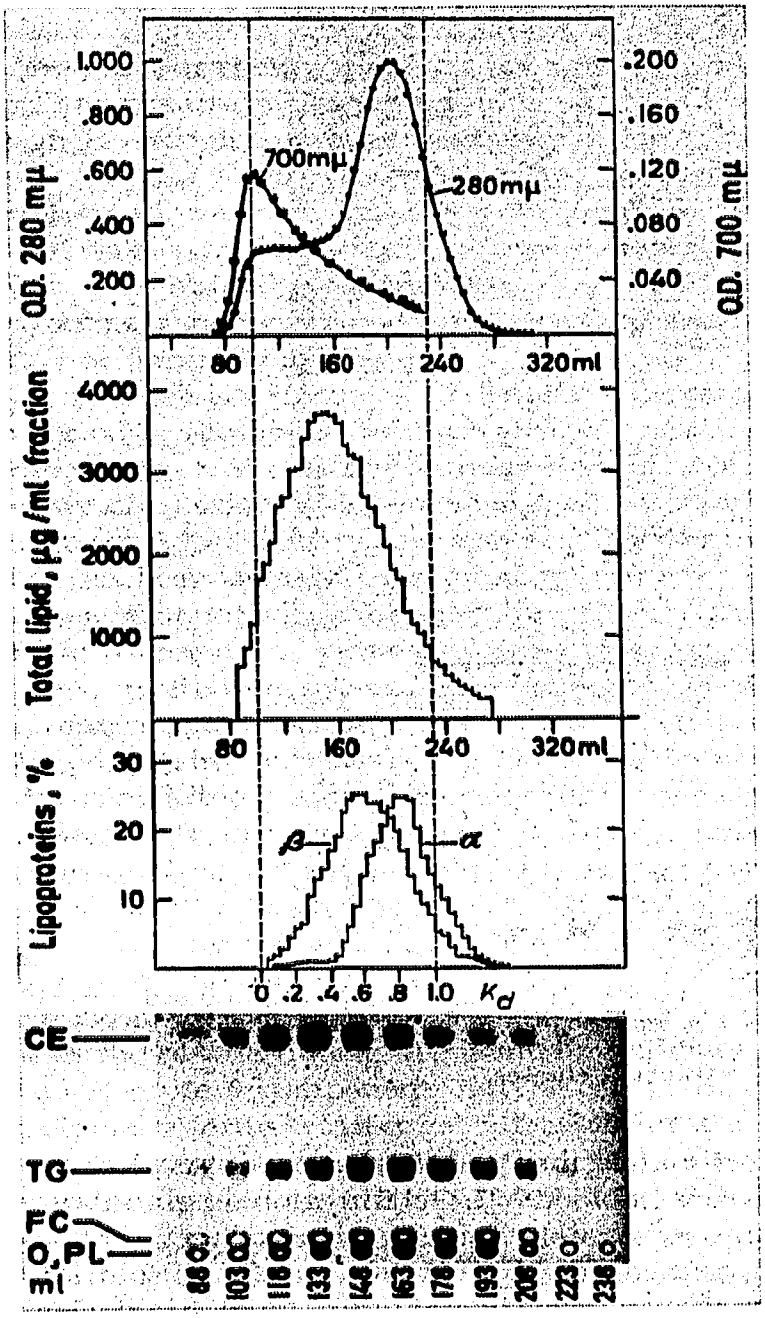

Fig. 4. Chromatography of 10 ml fasting serum of a woman with hyperlipemia (total lipids 3700 mg %) on a column of 2 % agarose beads. Optical densities at 280 mμ (eluates diluted 1:10), and 700 mμ. Total lipid content of eluates. α_1-Lipoprotein and β-lipoprotein concentrations are expressed in per cent of a standard serum. Thin-layer chromatography of lipid extracts of eluates (CE = cholesterol esters; TG = triglycerides; FC = free cholesterol; PL = phospholipids; O = origin).

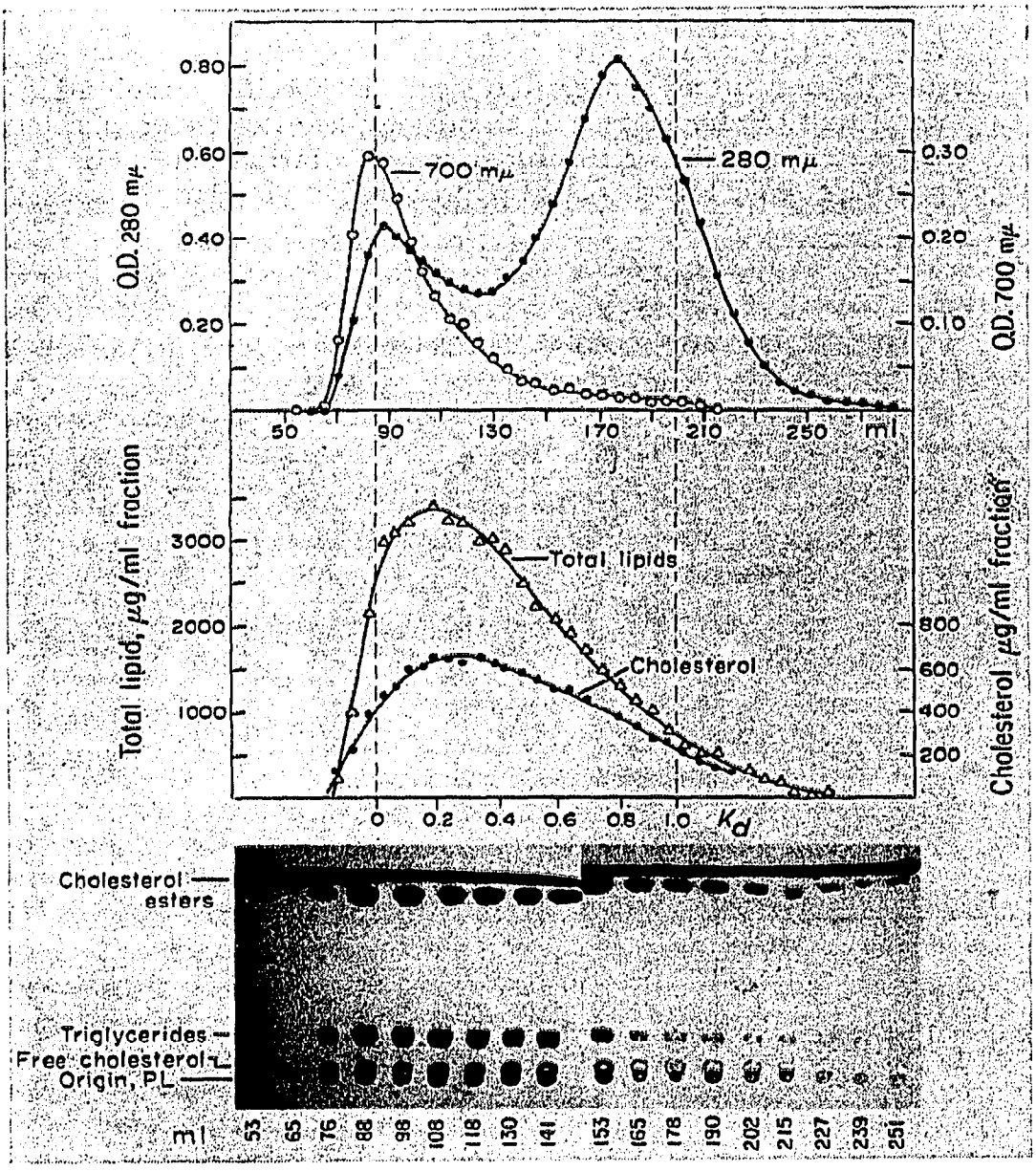

Fig. 5. Chromatography of 8 ml fasting serum of a patient with carbohydrate induced hyperlipemia (total lipids 3900 mg %) on a column of 2 % agarose beads. Top: optical densities at 280 mμ (eluates diluted 1:10) and 700 mμ. Middle: total lipid and total cholesterol content of eluates. Bottom: thin-layer chromatography of lipid extracts of eluates.

techniques have been devised[13–15]. Unfortunately many of these procedures alter the native state in which lipoproteins are present in blood[16, 17]. Gel filtration on granulated agarose offers an alternative method for lipoprotein fractionation[1]. The sample is subjected only to the forces of diffusion, and constantly remains in buffered solution. Varying patterns of lipoprotein distribution in hyperlipemia can be distinguished by this method (Figs. 3–5).

There is no general agreement on whether proteins are integral components of the chylomicron surface or whether they should be considered contaminants[16]. Centrifugation in saline or buffer can remove almost all the nitrogen from the larger

fat particles[18], prolonged exposure to serum on the other hand alters the amount and composition of their surface proteins[19,20]. After gel filtration none of the following proteins could be demonstrated by radial immunodiffusion at the front, where large particles elute: β-lipoprotein, α_1-lipoprotein, α_2-macroglobulin, acid α_1-glycoprotein, antitrypsin, γG-globulin. These findings are in some contrast with the assumption that, in the blood stream, small amounts of α_1- and β-lipoproteins form a complex with the lipids absorbed in the intestine (primary particles)[21]. If gel filtration does not destroy these complexes, their content of α_1- or β-lipoproteins was either too small for detection, or their antigenic sites were masked. The possibility that the chylomicrons do not penetrate the gel in immunodiffusion, and that they hold the surface proteins tightly enough to prevent their free diffusion also has to be considered.

It has recently been suggested that α_1-lipoproteins combine with β-lipoprotein to form the very low density lipoproteins ($S_f > 20$, density < 1.006, pre-β-lipoproteins) that take part in the transport of endogenous triglyceride[22]. The serum analyzed in Fig. 4 contained large amounts of very low density lipoproteins. In this case α_1-lipoproteins were immunologically detectable in small amounts up to the front, where they do not normally appear.

There is agreement that the low density lipoproteins (S_f 0 to 20, density 1.006 to 1.063, β-lipoproteins) are antigenically distinct from high density lipoproteins (density $>$ 1.063, α_1-lipoproteins)[23,24]. In spite of wide physicochemical heterogeneity, complete antigenic homogeneity of low density lipoproteins[24,28] has been demonstrated. A low density lipoprotein component with a hydrated density of 1.018 ($S_f >$ 12) has been isolated from human serum[29,30]. This fraction, called α_2-lipoprotein after its electrophoretic mobility on starch or polyvinyl chloride, has been differentiated from β-lipoprotein. Immunologically, however, the two cannot be distinguished[31,32]. Since α_2-lipoprotein contains 8% polypeptide, and β-lipoprotein 16.5%[32] the immunological reaction of β-lipoprotein is relatively stronger. A skewed curve of β-lipoproteins with tailing to high K_d values was often found by radial immunodiffusion after gel filtration on 2% agarose beads. Plates of 2% agarose were usually chosen to avoid steric hindrance of diffusion of β-lipoproteins during immunoprecipitation, but identical patterns were found with 1% and 3% concentrations. Since agar interacts with low density lipoproteins[33], agarose should be used exclusively.

In contrast to plasma low density lipoproteins the high density lipoproteins (density 1.063 to 1.21, α_1-lipoproteins) are immunologically heterogeneous[10,17,34–36]. Two forms αLP_A, and αLP_B, have been distinguished, the first being transformed into the second during ultracentrifugation and storage[36]. Antigenic polymorphism of the α_1-protein component may be due to the presence in solution of monomeric and polymeric forms[10]. After gel filtration on 2% agarose beads α_1-lipoproteins consistently appeared as a symmetrical peak. Double rings observed in immunodiffusion may be due to conversion of αLP_A into αLP_B. They were not produced consistently by the same antiserum, and appeared mostly in aged samples. Such conversions may also be the cause of the difficulty encountered in producing a stable standard serum for calibration of immunological methods[37].

SUMMARY

Estimation of plasma lipoproteins, and other proteins by radial immunodiffu-

sion after gel filtration, is described. Gels of 2 and 4 % granulated agarose were used. On 2 % granulated agarose, large chylomicrons elute with the front, very low density lipoproteins (S_f 20–400) at K_d values up to 0.45, low density lipoproteins (β-lipoproteins; S_f 0–20) at K_d 0.50 to 0.62, and high density lipoproteins (α_1-lipoproteins) at K_d 0.77 to 0.85. The latter values confirm those predicted from calibration, proving that lipoproteins are not appreciably adsorbed to the bed material. Different forms of hyperlipemia can be distinguished by this method.

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