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Affinity Chromatography of Human Plasma Low- and High-Density Lipoproteins

ELUTION BY SELECTIVE CLEAVAGE OF A BOND IN THE SPACER

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Human plasma low- and high-density lipoproteins were found to bind to Sepharose gels containing coupled cholesterol or cholic acid. The lipoproteins were bound very strongly, and it was not possible to elute them under non-denaturing conditions. The detergents Triton X-100 and sodium dodecyl sulphate eluted the lipoproteins in partly denatured form. Adsorbents were used where the steroid was coupled through a spacer containing a thiol ester bond. It was thus possible to elute bound lipoproteins by selective cleavage of the bond with hydroxylamine. A small proportion of albumin was the only contaminant detected, the amounts depending on which ligand was used. Low- and high-density lipoproteins were separated by gel filtration. They behaved as did the native molecules when analysed by gel filtration, immunodiffusion, immunoelectrophoresis and electrophoresis in polyacrylamide gradient gels. The high capacity and the selectivity of the adsorbents make them suitable for the removal of lipoproteins from protein solutions.

The function of the plasma lipoproteins is to solubilize and transport lipids. They also take part in the regulation of some enzymes. Several reviews have been published concerning the structure and function of the lipoproteins (e.g. Lloyd & Fosbrooke, 1974; Scanu *et al.*, 1975; Day & Levy, 1976; Smith *et al.*, 1978). The low density of the lipids results in lower buoyant densities of the plasma lipoproteins than of the other plasma proteins. This, and differences in lipid content, have made it possible to define the following lipoprotein classes, namely HD lipoprotein, LD lipoprotein and VLD lipoprotein. The isolation of these lipoprotein classes is usually obtained by fractionated flotation in the ultracentrifuge.

During plasma-protein fractionation the lipoproteins are enriched in certain fractions. The high proportion of lipoproteins makes it difficult to use these fractions as source for other proteins.

In the present study, affinity-chromatography adsorbents were developed for the removal of LD and of HD lipoprotein from plasma. It was also possible to obtain purified LD and HD lipoprotein. The lipoproteins bind various lipids such as triacylglycerols, phospholipids and cholesterol. Of the several alternative ligands that could be tried, cholesterol and cholic acid were chosen, mainly because they are probably not bound to other plasma proteins to any large extent. Adsorbents containing these ligands showed strong affinity for LD and HD lipoprotein. The lipoproteins could be desorbed either by using detergents or by selective cleavage of certain bonds in the spacer.

Abbreviations used: HD lipoprotein, high-density lipoprotein with density 1.063–1.21 g/ml; LD lipoprotein, low-density lipoprotein with density 1.006–1.063 g/ml; VLD lipoprotein, very-low-density lipoprotein with density <1.006 g/ml.

Materials and Methods

Cholesterol (5-cholesten-3β-ol) hydrogen succinate and cholic acid were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., CNBr and dicyclohexylcarbodi-imide from Fluka A.G., Buchs, Switzerland, cystamine dihydrochloride and 2,2′-dithiodipyridine from Sigma, St. Louis, MO, U.S.A., and 1,6-diaminohexane and hydroxylamine hydrochloride from Merck, Darmstadt, Germany. The detergents Triton X-100 and sodium dodecyl sulphate were purchased from Kebo AB, Stockholm, Sweden. Sephadex and Sepharose gels were from Pharmacia Fine Chemicals, Uppsala, Sweden, and agarose, batch AGS 019A, from Litex, Glostrup, Denmark. A mixture of acrylamide monomers (Cyanogum 41) was obtained from Fisher Scientific Company, Pittsburgh, PA, U.S.A. Gradient polyacrylamide (Gradipore) gels were from Universal Scientific through AB Lamda, Stockholm, Sweden. Precoated t.l.c. plates (Silica-gel 60) were purchased from Merck. Rabbit antisera against LD lipoprotein [anti-(β-lipoprotein) serum] and HD lipoprotein

[anti-(α_1-lipoprotein) serum] were obtained from Svenska Hoechst AB, Stockholm, Sweden. All other chemicals were of the best available commercial grade.

Preparation of the adsorbents

The adsorbents were prepared by first coupling a spacer (1,6-diaminohexane or cystamine) to the gel and then attaching the ligand to the spacer. The CNBr method (Axén *et al.*, 1967) was used to couple the spacers to Sepharose 4B (Fig. 1). The activation was performed by using 1g of CNBr to 25ml of gel and the pH was maintained at 11 for 8min. The activated and washed gel was added to a solution of the spacer to be coupled. The 1,6-diaminohexane solution consisted of 1.6g dissolved in 10ml of water, and the pH was adjusted to 10 with 12M-HCl. This pH was maintained during the reaction. The cystamine solution was made from 0.45g of cystamine dihydrochloride and 15ml of 0.1M-sodium carbonate buffer, pH8.5. The reaction mixture was stirred for 18h at 22°C. The gel was then washed extensively.

Cholic acid and cholesterol hydrogen succinate were coupled to 1,6-diaminohexane- and cystamine-Sepharose by using dicyclohexylcarbodi-imide. The gels were washed extensively with water, dioxan, 10% (v/v) triethylamine in dioxan and dioxan again. The washed gel was added to a stirred solution containing 0.5g of cholic acid dissolved in 100ml of dioxane. The reaction was started by adding 0.35g of dicyclohexylcarbodi-imide dissolved in 1.0ml of dioxan. The coupling was performed with stirring at 22°C. After 2h a further portion of dicyclohexylcarbodi-imide was added, and 4h later the coupled gel was washed extensively with dioxan, water and finally with 0.1M-sodium phosphate buffer, pH7.4. Cholesterol hydrogen succinate was coupled in the same way, except that 0.5g was dissolved in 25ml of dioxan.

The adsorbents containing a thiol ester bond were prepared as follows. Cysteamine-Sepharose was obtained by reduction of cystamine-Sepharose with 0.05M-mercaptoethanol in 0.1M-sodium carbonate buffer, pH8.5, for 1h. The gel was washed extensively with the carbonate buffer, water and dioxan. To a reaction mixture of 0.65g of cholic acid dissolved in 130ml of dioxan were added 2ml of dioxan containing 0.54g of dicyclohexylcarbodi-imide and 25ml of cysteamine-Sepharose. After 2h with stirring at 22°C the same amounts of cholic acid in dioxan and dicyclohexylcarbodi-imide were added; 2h later the reaction was stopped by washing the gel with dioxan. The reaction procedure was repeated and was followed by extensive washing with dioxan, water and 0.1M-sodium carbonate buffer, pH8.5. Remaining thiol groups were blocked by treating the gel with 60mg of iodoacetamide for 45min. Finally, the adsorbent was washed with the carbonate buffer

(*a*) Sepharose—NH—$[CH_2]_6$—NH—

(*b*) Sepharose—NH—CH_2—CH_2—S—S—CH_2—CH_2—NH—

(*c*) Sepharose—NH—CH_2—CH_2—S—

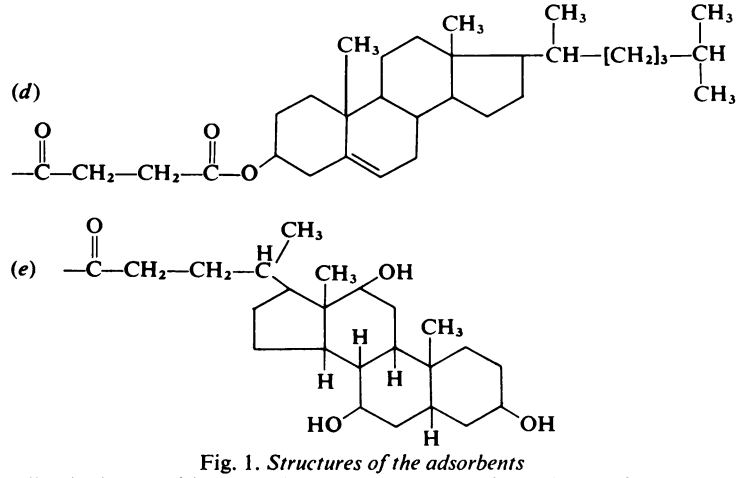

Fig. 1. *Structures of the adsorbents*

The spacers were 1,6-diaminohexane (*a*), cystamine (*b*) and cysteamine (*c*). To these were coupled cholesterol hydrogen succinate (*d*) and cholic acid (*e*).

and 0.1M-sodium phosphate buffer, pH7.4. Cholesterol hydrogen succinate was coupled in the same way, except that 0.65g was dissolved in 65ml of dioxan.

The coupling of 1,6-diaminohexane to the gel was verified by the trinitrobenzenesulphonate reaction (Cuatrecasas, 1970). The amount of coupled cystamine was determined by measuring the thiol content of the gel after reduction with 0.05M-mercaptoethanol as described above. The gel was washed extensively and the thiol content was determined by use of 2,2'-dithiodipyridine (Grassetti & Murray, 1967). To 0.5ml of gel was added 0.1M-sodium carbonate buffer, pH8.5, to give 3.0ml; 2.0ml of a saturated solution of 2,2'-dithiodipyridine in water was then added. The tube was rotated lengthwise for 15min and then centrifuged at 1000*g* for 10min at 22°C. The A_{343} of the supernatant was measured against an appropriate control. A molar absorption coefficient of 7060 litre·mol^{-1}·cm^{-1} of the reaction product, 2-thiopyridone, was used. The gels had 2–4μmol of thiol groups/ml of sedimented gel. The thiol content of the cysteamine–gel was decreased by about 0.6μmol of thiol groups/ml after coupling of steroid. After iodoacetamide treatment less than 0.02μmol of thiol groups/ml remained.

Analytical procedures

The quantification of HD and LD lipoprotein was performed by electroimmunoassay (Weeke, 1973) by using plasma pooled from 12 healthy donors as standard. The donors were six male and six female Caucasians, and plasma was collected after fasting, with their informed consent. In the determination of LD lipoprotein the S.E.M. was ±3.6, and of HD lipoprotein, ±2.8. An example of electroimmunoassay is shown in Fig. 2. The same technique was also used to locate LD and HD lipoprotein in the fractions obtained by gel filtration. The purity of different fractions was tested by immunoelectrophoresis essentially as described by Scheidegger (1955) and by electrophoresis in gradient polyacrylamide gel (Gradipore electrophoresis), as described in the manufacturer's instructions. The Gradipore electrophoresis was run for 18h at 100V in a pH8.3 buffer containing 89mM-Tris, 82mM-boric acid and 2.5mM-Na_2EDTA.

Ouchterlony double-immunodiffusion was performed in 1% (w/v) agarose. After development of the precipitates for 48h, the gels were treated and stained as after electroimmunoassay (Weeke, 1973).

Polyacrylamide-gel electrophoresis in 5 or 7.5% (w/v) gels was carried out in the presence of urea. Urea was dissolved in 0.2M-Tris/HCl, pH8.9, to give a concentration of 8M, and this buffer was used throughout. However, the samples were dissolved in an 8M-urea buffer made from 0.05M-Tris/HCl, pH8.9. Sucrose and Bromophenol Blue were added to the samples. The electrophoresis was run at 3mA/tube until the Bromophenol Blue band had migrated 6cm. The gels were stained with Coomassie Blue in 12.5% (w/v) trichloroacetic acid (Chrambach *et al.*, 1967) and destained in 10% (v/v) acetic acid.

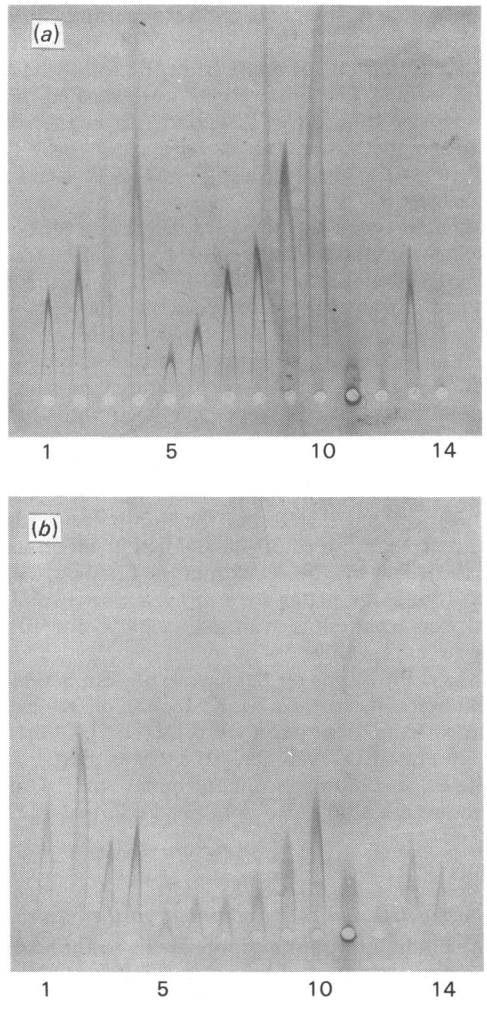

Fig. 2. *An example of electroimmunoassay of HD and LD lipoprotein*

The samples from various fractions were applied in wells 1–4 and 11–14. The plasma standard was applied in wells 5–10. The agarose contained 2% antiserum against HD (*a*) and LD (*b*) lipoprotein. The following dilutions of the plasma standard were used: (*a*) 5, 1:200; 6, 1:100; 7, 1:50; 8, 1:40; 9, 1:20; and 10, 1:10. The standards in 5–8 were used to construct the calibration curve. (*b*) 5, 1:100; 6, 1:50; 7, 1:40; 8,1: 20; 9, 1:10; and 10, 1:5. (Sample 11 had previously been exposed to sodium dodecyl sulphate and quantification was not possible.)

The isolated lipoproteins were compared with lipoproteins that had been obtained by the method of Rudel *et al.* (1974) apart from the following two modifications. The lipoproteins were washed in the high-density solution in a second run in the ultracentrifuge, because a fixed-angle rotor head was used, and the gel filtration was performed on Sepharose 6B.

The lipids were extracted from LD and HD lipoprotein with ethanol/acetone (1:1, v/v) after freeze-drying (Rudman *et al.*, 1968). Extraction from HD lipoprotein was also performed with ethanol/diethyl ether (2:3, v/v) by the method of Shore & Shore (1962), with the modifications described by Sohdi *et al.* (1971). The lipids were separated by t.l.c. on silica-gel plates, which were developed with diethyl ether and dried at 110°C before the samples were applied. The polar lipids were separated in chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) (Graveland, 1968) and the non-polar lipids in light petroleum (b.p. 30–40°C)/diethyl ether/acetic acid (90:10:1, by vol.) (Feldman & Fosslien, 1971). After drying, the plates were sprayed with a solution of copper acetate and warmed at 200°C for 10min (Fewster *et al.*, 1969).

The gel filtrations on Sepharose 6B and Sephadex G-200 were performed in K 16/100 or K 26/100 columns from Pharmacia. A 0.1M-Tris/HCl/0.15M-NaCl/0.5mM-EDTA buffer, pH8.0, was used.

The 0.1M-sodium phosphate buffer, pH7.4, used in the present study also contained 0.25mM-EDTA.

Results

Binding of LD and HD lipoprotein to the adsorbents

The binding of plasma lipoproteins to the various adsorbents was studied by using a batch technique. Separate pools of plasma were used in each adsorption experiment. The adsorption was started by mixing 20ml of adsorbent, 20ml of 0.1M-sodium phosphate buffer, pH7.4, and 80ml of plasma. The mixture was stirred for 2h at 4°C and then the supernatant was assayed for LD and HD lipoprotein. The difference in lipoproteins in the supernatant compared with the starting material was assumed to be the amount bound. Table 1 shows the results obtained with various gels. The gels without ligands (the spacer gels) did not bind any measurable amount of LD or HD lipoprotein. The binding of lipoproteins to the adsorbents therefore depends on coupled cholesterol or cholic acid and not on the spacer. The results in Table 1 show that the cholic acid adsorbents bound most of both LD and HD lipoprotein present, whereas the cholesterol adsorbents bound about 75% of applied HD lipoprotein and 20% or less of LD lipoprotein.

An estimate of the capacities of the adsorbents can be made by using a value of 3.0mg of HD lipoprotein and 3.7mg of LD lipoprotein/ml of plasma (Shore & Shore, 1972). The cholesterol and cholic acid adsorbents bound about 10 and 20mg of lipoproteins/ml of sedimented gel respectively. Thus the adsorbents have high capacities. The adsorbed gels turned yellow, especially the cholic acid adsorbents.

Table 1. *Binding of LD and HD lipoprotein to various substituted gels*

The adsorbents were stirred with plasma for 2h at +4°C. The supernatant after adsorption was assayed for LD and HD lipoprotein. It was assumed that the lipoproteins not found in the supernatant were bound to the adsorbents. The amount bound is expressed as a percentage of available LD or HD lipoprotein. Results are means ± S.E.M. for three experiments.

Ligand	Spacer	Lipoprotein bound	
		HD	LD
Cholesterol	1,6-Diaminohexane	87±7.8	21±9.5
	Cystamine	72±7.3	~0
	Cysteamine	64±10	~0
Cholic acid	1,6-Diaminohexane	92±2.6	78±3.2
	Cystamine	92±4.0	87±4.4
	Cysteamine	51±3.0	78±2.7
No ligand	1,6-Diaminohexane	~0	~0
	Cystamine	~0	~0
	Cysteamine–iodoacetamide	~0	~0

Desorption by detergents

Buffers of various compositions were used in attempts to elute the adsorbed lipoproteins from the cholesterol–diaminohexane–Sepharose. Elution with 1M-NaCl in 0.1M-sodium phosphate buffer, pH7.4, was found to elute some proteins, but not the lipoproteins. This step was subsequently used as a washing step before any other elution was tried. No lipoproteins were eluted with 5mM-sodium phosphate buffer, pH7.4, with or without 20% (v/v) ethanol. Use of the 0.1M-sodium phosphate buffer, pH7.4, saturated with cholic acid was also without effect. The buffers containing 0.05M-sodium phosphate buffer, pH7.4, 50% (v/v) ethylene glycol and either 1M-NaCl or 4mM-cholic acid eluted only small amounts of adsorbed proteins. The same results were obtained when the buffers were tested on the cholic acid–diaminohexane–Sepharose.

Desorption was possible, however, by using 0.1M-sodium phosphate buffer, pH7.4, containing 1% (w/v) Triton X-100 or 1% (w/v) sodium dodecyl sulphate. The lipoproteins had been eluted essentially quantitatively, because after extensive washing to remove the detergents the adsorbents bound the same amount of lipoproteins as before.

The eluted fractions were concentrated by low-pressure dialysis. Immunodiffusion of the fractions showed identity between HD lipoprotein in the eluates and HD lipoprotein in plasma. No precipi-

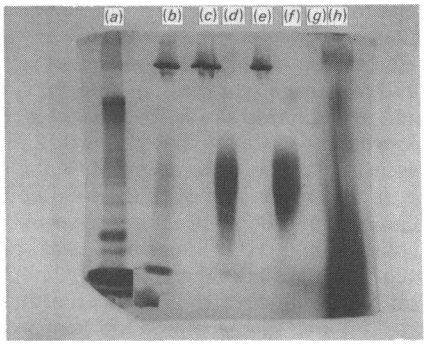

Fig. 3. *Gradipore electrophoresis of LD and HD lipoprotein and fractions eluted from adsorbents*

The samples are: plasma (*a*), eluate obtained from cholic acid–cysteamine–Sepharose by hydroxylamine (*b*), purified LD (*c*) and HD (*d*) lipoprotein obtained from eluate in (*b*), LD (*e*) and HD (*f*) lipoprotein obtained by ultracentrifugation, and eluate obtained by desorption from steroid–diaminohexane–Sepharose with Triton X-100 (*g*) and sodium dodecyl sulphate (*h*).

tates were obtained between antiserum against LD lipoprotein and eluates obtained with detergents. In Gradipore electrophoresis the eluted lipoproteins appeared to be denatured (Fig. 3). A broad diffuse zone was obtained in a position indicating a lower-molecular-weight range than for the native proteins. On gel filtration on Sepharose 6B this material gave an elution profile that was completely different from the one obtained with the native molecules. Thus no native lipoproteins were obtained by this procedure.

Desorption by selective cleavage of a bond in the spacer

To avoid formation of lipoprotein–detergent complexes, another approach was tried, namely elution by selective cleavage of a bond in the spacer. The lipoprotein and ligand would thus be eluted together in a complex. Two different types of cleavable bonds were used. In one the spacer contained a disulphide bond that could be broken by exposure to mercaptoethanol. In the other type the ligand was linked to the spacer through a thiol ester bond that could be cleaved by treatment with hydroxylamine (Cuatrecasas, 1970).

Cholesterol hydrogen succinate and cholic acid were coupled to cystamine–Sepharose as described above to give adsorbents that contained a disulphide bond in the spacer. The gels were mixed with plasma as described above and they bound normal amounts of LD lipoprotein and HD lipoprotein (see Table 1). After washing with 1 M-NaCl in the phosphate buffer, the gels were equilibrated with 0.1 M-sodium carbonate buffer, pH 8.5. Carbonate buffer was added to 10ml of sedimented gel to give a final volume of 25ml, and mercaptoethanol was added to give a concentration of 0.1 M. The mixture was stirred for 1h at 22°C. Elution was performed in a column and the adsorbent was washed with 20ml of the carbonate buffer containing mercaptoethanol. From the cholic acid adsorbent all yellow material was eluted, but not, however, from the cholesterol adsorbent. By electroimmunoassay it was found that 15% of the adsorbed lipoproteins were eluted from the cholesterol adsorbent and more than 90% from the cholic acid adsorbent.

The adsorbents containing a thiol ester bond were prepared by coupling the ligands to cysteamine–Sepharose as described above. The results from adsorption of lipoproteins to these gels are found in Table 1. The cholesterol adsorbent bound lipoproteins as did the other cholesterol adsorbents having other spacers. The cholic acid adsorbent bound slightly less LD lipoprotein and only about half the amount of HD lipoprotein bound by the other adsorbents containing cholic acid. The adsorbents were washed with 1 M-NaCl in the 0.1 M-sodium phosphate buffer, pH 7.4. After NaCl had been washed away, 10ml of sedimented gel was mixed with phosphate buffer containing sufficient hydroxylamine to give 1 M in 40ml of reaction mixture. Hydroxylamine hydrochloride had been dissolved in 0.1 M-sodium phosphate buffer and pH had been adjusted back to 7.4 with solid NaOH. After stirring for 2h at 22°C the gel was packed in a column and eluted. About 20ml of phosphate buffer containing 1 M-hydroxylamine was used to wash the gel. The eluate and washing were pooled and desalted on Sephadex G-25 in the phosphate buffer. The protein peak was collected and concentrated by low-pressure dialysis. Quantification of the eluted material by electroimmunoassay showed that about 9% of adsorbed HD lipoprotein was eluted from the cholesterol adsorbent. The corresponding eluate from the cholic acid adsorbent contained 30% of adsorbed HD lipoprotein and 70% of adsorbed LD lipoprotein. A further treatment of the gel with hydroxylamine yielded additional 9 and 8% of adsorbed HD and LD lipoprotein respectively. Two adsorbents with 0.6 μmol of cholic acid bound/ml of sedimented gel were found to contain 0.2 and 0.3 μmol of thiol groups/ml respectively after elution with hydroxylamine.

Studies on the lipoproteins obtained after selective cleavage

The lipoproteins eluted by mercaptoethanol from the adsorbents with cystamine as spacer were not stable. They started to precipitate a few hours after elution. Addition of iodoacetamide to block all thiol groups did not prevent the precipitation. LD and HD lipoprotein were quantified by starting the electroimmunoassay immediately after elution and thus before any precipitation was observed. No further work was done with this material.

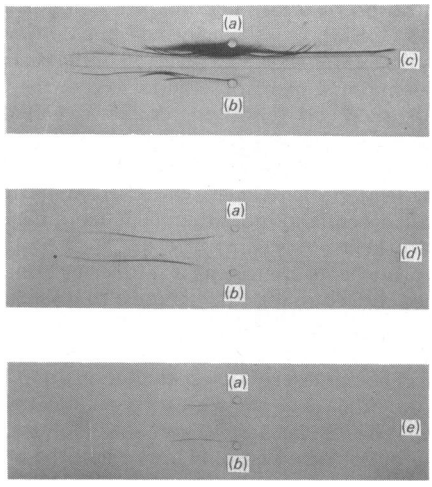

Fig. 4. *Immunoelectrophoretic comparison of an eluate with plasma*

The samples are (*a*) plasma, and (*b*) eluate, obtained from cholic acid–cysteamine–Sepharose after cleavage with hydroxylamine. Antisera against human serum proteins (*c*), HD lipoprotein (*d*) and LD lipoprotein (*e*) were used. The third precipitate in sample (*b*) contains albumin.

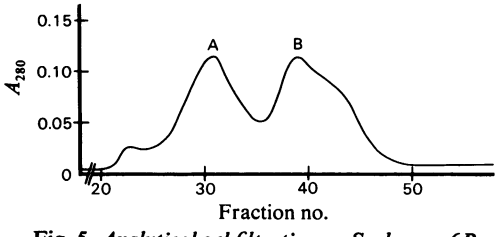

Fig. 5. *Analytical gel filtration on Sepharose 6B*

The sample was an eluate from cholic acid–cysteamine–Sepharose. Peak A contains LD lipoprotein and peak B HD lipoprotein. The shoulder on the descending slope of peak B comes from the albumin present in the sample. The gel bed had a diameter of 16mm and a length of 90cm. The flow rate was 12ml/h. The buffer was 0.1M-Tris/HCl/0.15M-NaCl/0.5mM-EDTA, pH8.0.

The fractions obtained after elution with hydroxylamine and subsequent desalting were found to contain LD and HD lipoprotein and albumin on Gradipore electrophoresis (Fig. 3) and on immunoelectrophoresis (Fig. 4). In the latter method, specific antisera were used to identify the three detectable components. The lipoproteins migrated to the same position as did their counterparts in plasma. The eluate from the cholic acid–cysteamine–Sepharose contained only small amounts of albumin, whereas the eluate from the cholesterol adsorbent contained more albumin.

The eluates from cholic acid–cysteamine–Sepharose were used for further studies. The lipoproteins were separated by gel filtration on Sepharose 6B (Fig. 5). LD lipoprotein was eluted at $V_e/V_t = 0.52$ and HD lipoprotein at $V_e/V_t = 0.66$, where V_e is the elution volume of the protein and V_t is the total volume of the column. No protein peak was detected in the void volume, and HD lipoprotein was partially separated from the albumin present. The lipoproteins obtained by the ultracentrifugation procedure were separated on the same column of Sepharose 6B. LD and HD lipoprotein were eluted in the same positions as LD and HD lipoprotein obtained from the affinity-chromatography adsorbent. All fractions were concentrated by low-pressure dialysis. HD lipoprotein was finally separated from the last traces of albumin by gel filtration on Sephadex G-200 and concentrated as above.

LD and HD lipoprotein obtained from the affinity-chromatography procedure after gel filtration were compared with corresponding native proteins in plasma by immunodiffusion. The precipitates showed identity; no 'spur' formation could be detected (Fig. 6). Electrophoresis on Gradipore gels of the purified lipoproteins showed that no difference could be observed in the lipoproteins obtained by the two methods (Fig. 3).

Equal amounts of HD lipoprotein from the affinity-chromatography and the ultracentrifugation procedures were delipidated as described above, and

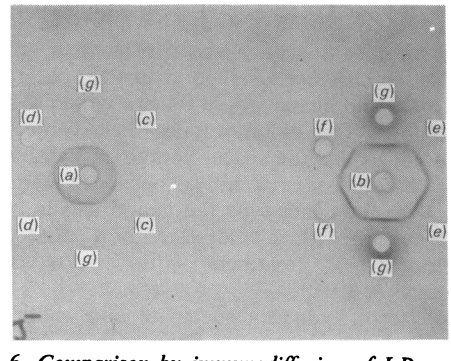

Fig. 6. *Comparison by immunodiffusion of LD and HD lipoprotein obtained by affinity chromatography with LD and HD lipoprotein in plasma*

LD and HD lipoprotein were purified by gel filtration from eluates obtained from cholic acid–cysteamine–Sepharose. The centre wells contained antisera against HD lipoprotein (*a*) and LD lipoprotein (*b*), respectively. HD (*c*, *d*) and LD (*e*, *f*) lipoprotein from two preparations as well as plasma (*g*) were placed in the peripheral wells.

the patterns of the lipids on the silica-gel plates were found to be identical. LD lipoprotein from the two procedures were compared in the same way and also there identical patterns were obtained. The apoproteins were compared by polyacrylamide-gel electrophoresis in the presence of 8M-urea. Apoproteins from HD lipoprotein were also obtained after extraction of lipids with tetramethylurea (Kane, 1973). No differences in band pattern could be observed whether the protein had been obtained by the affinity chromatography or the ultracentrifugation procedure.

The yields of LD and HD lipoprotein were determined in preparations starting from 80ml of plasma and 20ml of cholic acid–cysteamine–Sepharose. LD lipoprotein was obtained in a yield of 37% after the gel filtration and HD lipoprotein in 11% yield after both gel filtrations. The losses were made almost entirely in the adsorption and elution steps. However, no attempt was made to optimize the procedure.

Discussion

Cholesterol is readily incorporated into phospholipid monolayers and bilayers (Tanford, 1973) and lipoproteins (Margolis, 1969). Both free and esterified cholesterol are also natural components of lipoproteins. Cholesterol would thus be a possible ligand in an affinity-chromatography system for lipoproteins. In accordance with the expectations, experiments showed that adsorbents containing cholesterol did adsorb both LD and HD lipoprotein. Cholic acid was also tried as ligand to give an adsorbent containing a steroid with somewhat different structure and coupled through the other end of the molecule (Fig. 1). Under the conditions used it was found that the cholesterol adsorbents mainly bound HD lipoprotein, whereas the cholic acid adsorbents bound about equal amounts of LD and HD lipoprotein. As evident from the results obtained, both types of adsorbents showed strong binding of the lipoproteins, in fact too strong, as it was not possible to elute them under non-denaturing conditions.

These difficulties made it attractive to use a spacer–ligand system containing a bond that could be selectively cleaved. The experiments showed that it was possible to elute 30% of bound HD lipoprotein and 70% of bound LD lipoprotein from the cholic acid–cysteamine–Sepharose, and smaller amounts of bound lipoproteins were eluted from the cholesterol adsorbent. This difference between the adsorbents was also observed when cystamine was used as spacer and the disulphide bond was reduced with mercaptoethanol. Thus the bond to be cleaved is more accessible when cholic acid is used as ligand than when cholesterol is used. Furthermore, when the distance between the ligand and the bond to be cleaved was decreased, as in changing the spacer from cystamine to cysteamine, it was found that the amounts of eluted lipoproteins were decreased. However, the importance of changing elution conditions is not known.

It was thus possible to obtain LD and HD lipoprotein by affinity chromatography by using the adsorbent cholic acid–cysteamine–Sepharose. The LD and HD lipoprotein obtained after subsequent gel filtration were pure, and they behaved similarly to or identically with those in plasma or those obtained by ultracentrifugation in all respects tested. In order to use this procedure for purification of LD and HD lipoprotein, further characterization of them has to be made. It was not elucidated whether VLD lipoprotein was bound to the adsorbents, but the results indicate that no VLD lipoprotein was eluted from the adsorbent.

Lipoprotein contamination can often be a problem in connection with isolation of other proteins. This is a well-known problem in plasma-protein fractionation, e.g. lipoproteins cause problems during sterile filtration if present in the actual fraction. Further, they severely decrease the stability of the final protein solutions. In these cases it is necessary to remove the lipoproteins. It would be convenient to use an adsorbent with high specificity and high capacity to achieve a selective removal. Cholic acid–diaminoalkane–Sepharose could be such an adsorbent. Its properties are suitable as judged from the eluates from cholic acid–cysteamine–Sepharose. Lipoproteins have been found to bind to dodecylamine–Sepharose (Deutsch *et al.*, 1973), but such an adsorbent is not particularly specific. The steroid adsorbents can also be used to immobilize LD and HD lipoprotein. After 2 weeks at 4°C, no lipoproteins were found in the supernatant from an adsorbed cholesterol–diaminohexane–Sepharose.

Elution of proteins from affinity-chromatography adsorbents is usually obtained under relatively mild conditions. If the protein is strongly bound, it is desirable to use a spacer–ligand system containing a cleavable bond instead of using denaturing conditions for elution. In this way a protein–ligand complex will be obtained.

The cleavage procedure must be chosen not to modify the protein, and the bond to be cleaved must be accessible. Besides the method used in the present study, other methods have been published. Rosner & Smith (1975) used azodianiline as spacer and the azo bond was cleaved with dithionite. 20β-Hydroxy steroid dehydrogenase, which is stabilized by low-molecular-weight thiols, was eluted after reduction of a disulphide bond in the spacer with mercaptoethanol (Sweet & Adair, 1975). The latter method was tried in the present study, but disulphides in the proteins were probably also reduced, resulting in

aggregation and precipitation. A protein might be purified and complexed with a (labelled) ligand in a single step by these methods. If the complex can be eluted by reduction of a disulphide bond, it will contain a thiol group, which can be utilized for modification.

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