

The Isolation and Partial Characterization of the Serum Lipoproteins and Apolipoproteins of the Rainbow Trout

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1. VLD (very-low-density), LD (low-density) and HD (high-density) lipoproteins were isolated from the serum of trout (*Salmo gairdneri* Richardson). 2. Each lipoprotein class resembled that of the human in immunological reactivity, electrophoretic behaviour and appearance in the electron microscope. Trout LD lipoprotein, however, was of greater density than human LD lipoprotein. 3. The trout lipoproteins have lipid compositions which are similar to those of the corresponding human components, except for their high contents of long-chain unsaturated fatty acids. 4. HD and LD lipoproteins were immunologically non-identical, whereas LD lipoproteins possessed antigenic determinants in common with VLD lipoproteins. 5. VLD and HD lipoproteins each contained at least seven different apoproteins, whereas LD lipoprotein was composed largely of a single apoprotein which resembled human apolipoprotein B. 6. At least one, and possibly three, apoproteins were common to both VLD and HD lipoproteins. The major apoprotein of trout HD lipoprotein showed features which resemble human apoprotein A-1. 7. The broad similarity between the trout and human lipoprotein systems suggests that both arose from common ancestral genes early in evolutionary history.

Studies on the structure and metabolism of serum lipoproteins have been made predominantly on the human (Eisenberg & Levy, 1975; Scanu *et al.*, 1975) and rat (Bersot *et al.*, 1970; Koga *et al.*, 1971), though a number of other mammalian species have also been investigated (see Scanu *et al.*, 1975). Few detailed reports, however, have been made on the lipoproteins of non-mammalian species. Because of their more primitive evolutionary status and their special environmental situation, fish may have lipoproteins with features whose study might be of value in elucidating the processes involved in lipid transport in general.

The serum lipoproteins of several species of fish have been shown to consist of density classes which broadly resemble those of mammalian lipoproteins, yet they possess significant differences in their lipid moieties (Lauter *et al.*, 1968; Lee & Puppione, 1972; Mills *et al.*, 1977). Although the HD lipoproteins of the cod (Skinner, 1973) and the salmon (Nelson & Shore, 1974) have been reported to have polypeptide compositions that resemble those of their human

Abbreviations used: VLD, LD and HD lipoproteins; very-low-density, low-density and high-density lipoproteins respectively, of densities as defined in the text. S_0^0 and $F_{1.20}^0$ are flotation rates expressed in Svedbergs ($10^{13} \text{ cm} \cdot \text{s}^{-1} \cdot \text{dyn}^{-1} \cdot \text{g}^{-1}$) in NaCl and NaBr solutions of densities 1.063 and 1.20 g/ml respectively corrected for concentration-dependence and to 26°C.

counterparts, no data apart from preliminary reports (Skinner & Rogie, 1978; Chapman *et al.*, 1978) are available on the characterization of the apoproteins of VLD and LD lipoproteins of any fish species.

We have therefore isolated and characterized each of the major lipoprotein classes of the rainbow trout and have used a variety of techniques to investigate the structural relationships that exist between them. The results of these studies give an insight into the possible metabolic processes associated with the transport of lipid in the trout.

Materials and Methods

Chemicals

Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were purchased from Eastman Organic Chemicals (Kodak, Kirkby, Liverpool, U.K.). M.S. 222 (*m*-ethoxycarbonylanilinium methanesulphonate) was manufactured by Sandoz Products, London W.1, U.K. Oestradiol-17 β 3-benzoate dissolved in arachis oil/ethyl oleate containing 10% (v/v) benzyl alcohol and 0.3% cresol was from Intervet Laboratories, Bar Hill, Cambridge CB3 8EW, U.K. Freund's complete adjuvant and Noble agar were from Difco Laboratories, Detroit, MI, U.S.A. Silicic acid was obtained from Mallinckrodt, St. Louis, MO, U.S.A.

All other reagents and solvents were of analytical grade, purchased from BDH, Poole, Dorset, U.K. Aqueous solutions of urea were deionized immediately before use by passage through a mixed-bed ion-exchange resin [AG501-X8(D); Bio-Rad, Richmond, CA, U.S.A.].

Fish

Commercially farmed male trout (*Salmo gairdneri* Richardson) (1.0–1.6 kg) obtained from local fish farms were maintained in aerated fresh water at 10°C for 1–3 days before bleeding. The fish were offered standard fish pellets. Blood, removed from the caudal vein by means of a syringe, was allowed to clot at ambient temperature for 2–3 h. Serum was isolated by centrifugation at 1500g for 10 min at 10°C and re-centrifuged under the same conditions. Lipoprotein fractionation was started without delay. Serum from three to ten fish was pooled for the isolation of lipoproteins, except for experiments on the distribution of lipoproteins, in which case sera from individual trout were treated separately. For studies on the lipid composition of VLD lipoprotein, male fish were anaesthetized in fresh water containing 0.04% of M.S. 222 and injected intramuscularly with oestradiol-17 β 3-benzoate (2 mg/kg body wt.). The fish were allowed to recover in aerated fresh water before they were returned to the holding tank. After 7 days the fish were bled and the serum was prepared as described above.

Preparation of lipoproteins

Lipoproteins were prepared as follows by flotation at densities which were selected to yield pure products as judged by electrophoresis and immunodiffusion. All NaBr solutions contained 0.01% EDTA and densities were routinely measured at 20°C by pycnometry. No attempt was made to remove chylomicrons, if present, because of the small amount of material of $d < 1.020$.

Batches (10.4 ml) of serum (the density of which measured 1.020 g/ml) were centrifuged in 7.62 cm \times 1.58 cm polyallomer tubes in the Spinco model L-2 preparative ultracentrifuge in the type 40 rotor, for 18 h at 105 400g (40 000 rev./min) and at 10°C. The top 1 ml, containing VLD lipoprotein, was removed from each tube with the aid of a Beckman tube slicer. A second slicing was made at a point 2.8 cm from the top of the tube, and the middle fraction (approx. 5 ml) was discarded. The bottom fraction (approx. 4.4 ml) was mixed with an equal volume of NaBr solution of density 1.167 g/ml to give a solution density of 1.096 g/ml, and NaBr solution of this density was added to restore the volume to that of the original serum. A small gelatinous pellet that was present in most experiments was suspended in

the bottom fraction before adjusting the density. After centrifugation under the same conditions, the tube was sliced twice as previously. The upper 0.5 ml contained LD lipoprotein and the middle fraction was again discarded. The bottom fraction was adjusted to a density of 1.210 g/ml by addition of an equal volume of NaBr solution of density 1.326 g/ml and, after being made up to a volume of 10.4 ml with NaBr solution of density 1.210 g/ml, was centrifuged for 42 h at 105 400g (40 000 rev./min). The HD lipoprotein was recovered in the top 0.5 ml. The VLD lipoprotein and HD lipoprotein fractions were washed once by a second centrifugation in NaBr for 18 h at densities 1.020 and 1.210 g/ml respectively, and the LD lipoprotein was re-centrifuged at density 1.085 g/ml to remove any traces of HD lipoprotein.

To obtain quantitative yields of lipoproteins for determination of their distribution, the centrifuge tubes were sliced at only one point, approx. 2.5 cm from the top of the tube, after each centrifugation. The entire lower layer was adjusted to the appropriate density in the manner described, and the upper layer was analysed for lipoprotein without further washing.

For the isolation of total lipoproteins for the scanning of polyacrylamide gels, the density of serum was adjusted to 1.215 g/ml by the addition of an appropriate volume of NaBr solution of density 1.326 g/ml and centrifuged as described above for 42 h at 40 000 rev./min at 10°C.

Delipidization procedures

Lipoprotein fractions, after dialysis at 5°C against 0.15 M-NaCl/1 mM-EDTA, pH 8.0, were injected into 50 vol. of ethanol/ether [3:1 (v/v) for VLD lipoprotein and LD lipoprotein, and 3:2 (v/v) for HD lipoprotein] at -10°C (Scanu & Edelstein, 1971). After standing for 2 h at -10°C, the delipidized proteins were centrifuged for 10 min at 2000g and the pellets washed six times with ether. The first two supernatants were pooled and the resultant precipitate was combined with the main pellet for subsequent ether washes (Koga *et al.*, 1969). Apoproteins were stored at -20°C until used.

Electrophoresis

Agarose-gel electrophoresis was carried out by a procedure based on that of Bodman (1960). Agarose (0.7 g/100 ml) in veronal buffer, pH 8.6 (8.3 mM-diethylbarbituric acid / 41.6 mM-sodium diethylbarbiturate) was heated until molten and 6 ml volumes were pipetted on to glass slides (50 mm \times 75 mm). Perspex formers (5 mm long, 1 mm wide and 2 mm high) were inserted in the liquid gel at a position 2 cm from one end of the slide. After 30 min, the formers were carefully removed and the wells thereby

formed were filled with a 1/1 (v:v) mixture of agarose medium at 40°C and serum or appropriate lipoprotein solution. The slides were supported in an inverted position on filter-paper wicks and electrophoresis was carried out at 4°C for 45 min at 45 mA per plate. The slides were placed in 3% (w/v) trichloroacetic acid for 30 min and then in acetic acid/ethanol/water (1:8:11, by vol.) for a further 30 min. After drying in air, lipoprotein bands were detected by staining in Oil Red O at 37°C for 18 h.

Polyacrylamide-gel electrophoresis of lipoproteins was performed by using the system described by Davis (1964). Gels were made up of separating gel (of length 5.5 cm) containing 3.75% (w/v) acrylamide at pH 8.9 overlaid with a spacer gel (of length 1 cm) containing 2.5% (w/v) acrylamide at pH 6.8. Samples of serum or lipoproteins (after dialysis against 0.9% NaCl to remove NaBr) were applied in volumes of 20 µl containing sucrose. Apoproteins were analysed on 7.5% (w/v) acrylamide separating gels (of length 6.5 cm) containing 8 M-urea without the use of spacer gels. For both systems, electrophoresis was carried out at 2 mA per gel until the buffer front was within approx. 1 cm of the bottom of the tube. Bromophenol Blue was used as marker of the buffer front and the R_f value of the separated bands was expressed as the ratio of distance of migration to that of the dye front. Lipoproteins were detected by prestaining the samples with Sudan Black and protein was detected with Amido Black. Each sample was normally analysed in 2 or 3 gels containing a range of sample sizes varying from 10 to 200 µg to achieve a satisfactory resolution of heavy components while still detecting minor components that were present in only minute proportions. Polyacrylamide gels were scanned on a Polyfrac u.v. scanner fitted with a 280 nm filter (Joyce-Loebl, Team Valley, Gateshead, Tyne and Wear, U.K.).

Immunological methods

Antisera to trout serum and to HD lipoprotein were prepared by injecting rabbits intramuscularly with solutions of these emulsified with an equal volume of Freund's complete adjuvant. About 2–3 mg of protein in 1 ml of emulsion was injected initially and again after 1 week; 3 weeks later, a series of intravenous injections was given of a co-precipitate of antigen with Al(OH)₃. The rabbits were bled 5 days after the final injection and the antiserum was stored at –20°C.

For the preparation of antisera to trout VLD lipoprotein, LD lipoprotein and apo-(HD lipoprotein), the proteins were dissolved in 0.15 M-NaCl in which these components were completely soluble. For apo-(VLD lipoprotein), a suspension in 0.15 M-NaCl was used, since this apolipoprotein was only partially soluble. After emulsification of these

solutions or suspensions with equal volumes of Freund's complete adjuvant, volumes of 0.2–0.4 ml, containing 10–100 µg of protein, were injected into the exposed popliteal lymph nodes. After 1 month, a similar suspension was administered intramuscularly and the animals were bled 10 days later.

Two rabbits were used for each antigen. In every case the antisera produced from the two rabbits gave identical patterns when reacted with antigen by the double-diffusion technique of Ouchterlony (1949), in medium containing 1.25% (w/v) Noble agar, 0.9% NaCl and 0.01% thiomersal. In some instances, however, there was a small difference in the sharpness of the precipitin arcs produced. The antiserum that produced the sharpest arcs was selected for use.

Analytical ultracentrifugation

Lipoproteins were isolated by the procedure described above and dialysed against either NaCl solution of d 1.063 or NaBr solution of d 1.210 g/ml (measured at 20°C). Solutions were examined in plane and wedge-window cells in the Beckman model E ultracentrifuge at 52640 rev./min. Analyses were performed over a range of protein concentrations (HD lipoprotein, 2.5–10.0 mg/ml; LD lipoprotein, 5–10 mg/ml; VLD lipoprotein, 2.5–5.0 mg/ml) and no significant concentration-dependence of the flotation coefficients was obtained. The coefficients were corrected to standard conditions (see Abbreviations) as described by Lindgren *et al.* (1972).

Lipid and protein analysis

For lipid analysis, the lipoproteins were delipidized with chloroform/methanol (2:1, v/v) (Gustafson *et al.*, 1965) and the lipid classes fractionated on columns (8 mm × 140 mm) containing approx. 1.8 g of silicic acid by a modification of the method of Lis *et al.* (1961). A total of 10–20 mg of lipid was applied to columns through which were passed 15 ml volumes of 1.5, 8.0 and 55% (v/v) ether in light petroleum (b.p. 40–60°C) followed by methanol to elute cholesteryl ester, triacylglycerol, unesterified cholesterol and phospholipid respectively. The amount of each lipid was determined gravimetrically with a Stanton Unimatic CL 5D semi-micro balance (Oertling, Orpington, Kent, U.K.) with a recovery of 95–100%. To confirm that resolution of the above lipid classes had been achieved, the fractions obtained in each experiment were analysed, after weighing, by t.l.c. on silica gel G (Merck, Darmstadt, Germany) with cyclohexane/chloroform (1:1, v/v) and chloroform/methanol/acetic acid/water (50:25:8:4, by vol.) as developing solvents. Lipids were detected by spraying with H₂SO₄ and heating at 110°C. The effectiveness of the procedure was previously established by using artificial mixtures of cholesterol,

cholesteryl oleate, trioleoylglycerol and phosphatidylcholine as well as lipid extracts of fish lipoproteins. AnalaR grade solvents were used as they were shown in preliminary experiments to be as effective as the redistilled solvents.

For fatty acid analyses, the lipids were extracted from lipoprotein fractions by the method of Bligh & Dyer (1959) as modified by Allen *et al.* (1966). The lipid samples were transmethylated by the procedure of Hornstein *et al.* (1967) and the methyl esters separated as described by Cowey *et al.* (1976) by g.l.c. on columns containing EGSS-X and EGSS-Y (co-polymers of ethylene glycol succinate with a methylsilicone to provide phases of high and medium polarity respectively; Applied Science Laboratories Inc., State College, PA, U.S.A.). Identification of fatty acids was based on published values for retention times and comparison with known standards. After initial calibration with a wide range of standards and mixtures (Applied Science Laboratories), routine checks on retention times were made with mixtures of C_{18:1} and C_{22:4, ω6} acids.

Protein was determined by the method of Lowry *et al.* (1951) as modified by Miller (1959), with bovine serum albumin as standard.

Gel filtration

Samples of apo-(HD lipoprotein) and of apo-(LD lipoprotein), containing 20–30mg of protein in 3ml of eluting buffer, were applied to columns (2.2cm × 90cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), and eluted with 0.01 M-Tris/HCl (pH 8.6)/1 mM-EDTA/8 M-urea (Scanu *et al.*, 1969). The column effluent was continuously monitored on a LKB Uvicord 11 at 280nm and the A₂₈₀ of individual fractions was also read with a Unicam SP.500 spectrophotometer. Appropriate fractions were pooled and concentrated by ultrafiltration by dialysis against 0.15M-NaCl.

Amino acid analysis

Apoproteins were hydrolysed in evacuated tubes with 0.5ml of 5.5 M-HCl at 110°C for 20, 48 and 72 h. Quantitative analyses were performed on a single column (0.3cm × 100cm), eluted by an 8 h cycle of stepwise buffer changes, using the Locarte amino acid analyser (The Locarte Co., London W12 9RT, U.K.).

Results

Electrophoretic and immunological characteristics of isolated trout lipoproteins

Each of the fractions, VLD lipoprotein, LD lipoprotein and HD lipoprotein, isolated at $d < 1.020$, 1.020–1.085 and 1.096–1.210 respectively, migrated

as a single band on agarose gel electrophoresis (Fig. 1) and accounted for all the lipid-staining material present in unfractionated serum.

On polyacrylamide gels, the $d < 1.020$ fraction routinely showed a single band that penetrated only a short distance into the separating gel, with some material migrating a short distance ahead of it (Fig. 2). In some preparations, a second band, which stained for both protein and lipid, was also present at the junction of the spacer and separating gels. Subsequent observations suggested that this band represents chylomicrons. When an unstained gel was embedded in agar and a parallel channel subsequently cut and filled with anti-(VLD lipoprotein), each component reacted to give a single precipitation line, the two lines fusing to give a reaction of identity (Fig. 3). Polyacrylamide-gel electrophoresis of the fraction floating at $d 1.020$ – 1.085 showed the presence of two diffuse bands migrating close to each other, with a small amount of an additional component of slower mobility. Storage of the sample at -20°C or overloading of the gel resulted in the appearance of aggregated material at the origin and a failure to resolve the two major components. The two major components gave single precipitin arcs on reaction with anti-(LD lipoprotein), and a reaction of identity was shown to occur between them (Fig. 3). The $d 1.096$ – 1.21 fraction showed a sharp intense band at the position of the buffer front, together with a slower, more diffuse band; trailing material which stained for both protein and lipid was also present. Reaction

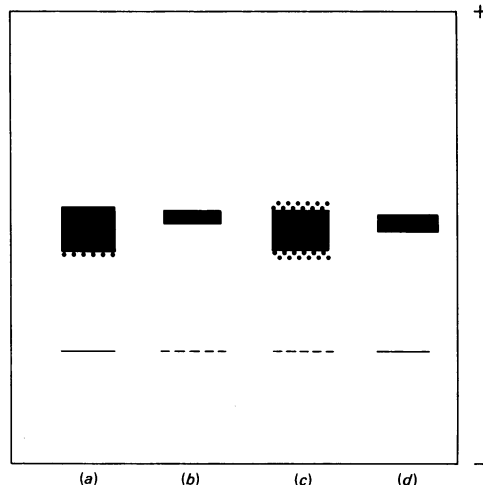


Fig. 1. Electrophoresis of trout serum lipoproteins on agarose

Proteins were submitted to electrophoresis on 0.7% agarose gels at pH 8.0 and stained for lipid with Oil Red O after drying. (a) Unfractionated trout serum; (b) HD lipoprotein; (c) LD lipoprotein; (d) VLD lipoprotein.

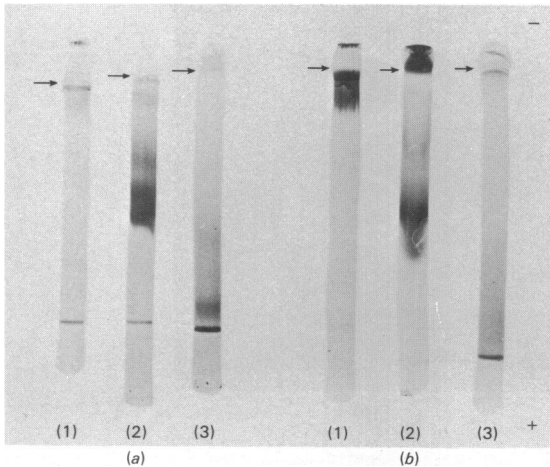


Fig. 2. Electrophoresis of trout serum lipoproteins on polyacrylamide gels

Separating gels containing 3.75% polyacrylamide were overlaid with 2.5% spacer gels at the positions indicated by arrows. Gels were stained for protein with Amido Black after electrophoresis (a) or prestained with Sudan Black for the detection of lipids (b). (1) VLD lipoprotein; (2) LD lipoprotein; (3) HD lipoprotein.

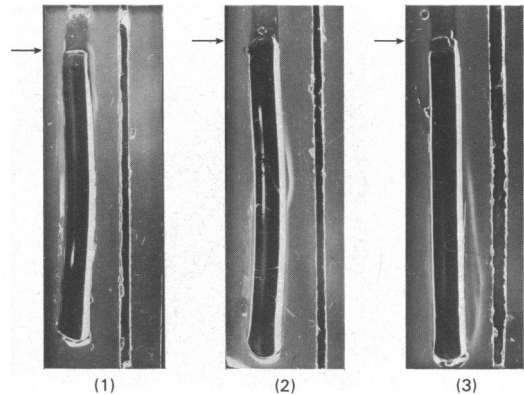


Fig. 3. Immunodiffusion patterns of trout serum lipoproteins after electrophoresis on polyacrylamide gels

After electrophoresis of the unstained lipoproteins, gels were embedded in 1.25% agar containing 0.9% NaCl and 0.01% thiomersal. Channels were cut parallel at a distance of 6 mm from the gel and filled with appropriate antibody. Precipitin arcs developed in 24–72 h. (1) Trout VLD lipoprotein was applied to the polyacrylamide gel and the channel was subsequently filled with rabbit anti-(VLD lipoprotein); (2) trout LD lipoprotein applied to gel and channel filled with anti-(LD lipoprotein); (3) trout HD lipoprotein applied to gel and channel filled with anti-(HD lipoprotein).

with anti-(HD lipoprotein) revealed the presence of two precipitin arcs, one of which was centred on the component nearest to the buffer band, whereas the other extended along the gel. A third precipitin line was observed in some preparations. The trailing components were also present in whole serum and were therefore not artifacts produced during the isolation procedure.

On the basis of the above results, it is considered valid to refer to the $d < 1.020$, $d 1.020$ – 1.085 and $d 1.096$ – 1.21 fractions as VLD, LD and HD lipoproteins respectively, with the implication that the VLD lipoprotein fraction contains a small proportion of chylomicrons.

The immunological inter-relationships between the three lipoprotein fractions are shown in Fig. 4. Anti-(VLD lipoprotein) serum gave two precipitin lines on reaction with VLD lipoprotein and two lines with LD lipoprotein; a faint line was given with some preparations of HD lipoprotein, and a reaction of identity was given between one of the lines produced with VLD lipoprotein and one with LD lipoprotein. Anti-(LD lipoprotein) serum produced four precipitin arcs on reaction with LD lipoprotein, two of which showed identity with two of the three arcs formed with VLD lipoprotein; no precipitin arcs were detected when anti-(LD lipoprotein) was tested with HD lipoprotein. Anti-(HD lipoprotein)

serum which has been shown to contain anti-(LD lipoprotein) activity gave three precipitin lines with HD lipoprotein; with VLD lipoprotein, one heavy and one very faint arc were produced. The relationship between the latter and the lines produced with HD lipoprotein were indistinct, though a reaction of identity with one of the HD lipoprotein arcs was suggested. Two precipitin lines were given on reaction of this impure anti-(HD lipoprotein) with LD lipoprotein, one of which showed a distinct reaction of identity with the heavy arc produced with VLD lipoprotein. The three lines formed with HD lipoprotein gave distinct patterns of non-identity with both the LD lipoprotein lines. When the anti-(HD lipoprotein) serum was adsorbed with LD lipoprotein, no precipitin lines were formed with either VLD or LD lipoproteins, whereas with HD lipoprotein at least two and possibly three precipitin lines developed, but rapidly diffused. These observations demonstrate that LD lipoprotein contains antigenic determinants in common with VLD lipoprotein, but that HD and LD lipoproteins are immunologically distinct.

The three lipoprotein fractions isolated in the preparative ultracentrifuge were also routinely tested with antibody raised against whole serum. The

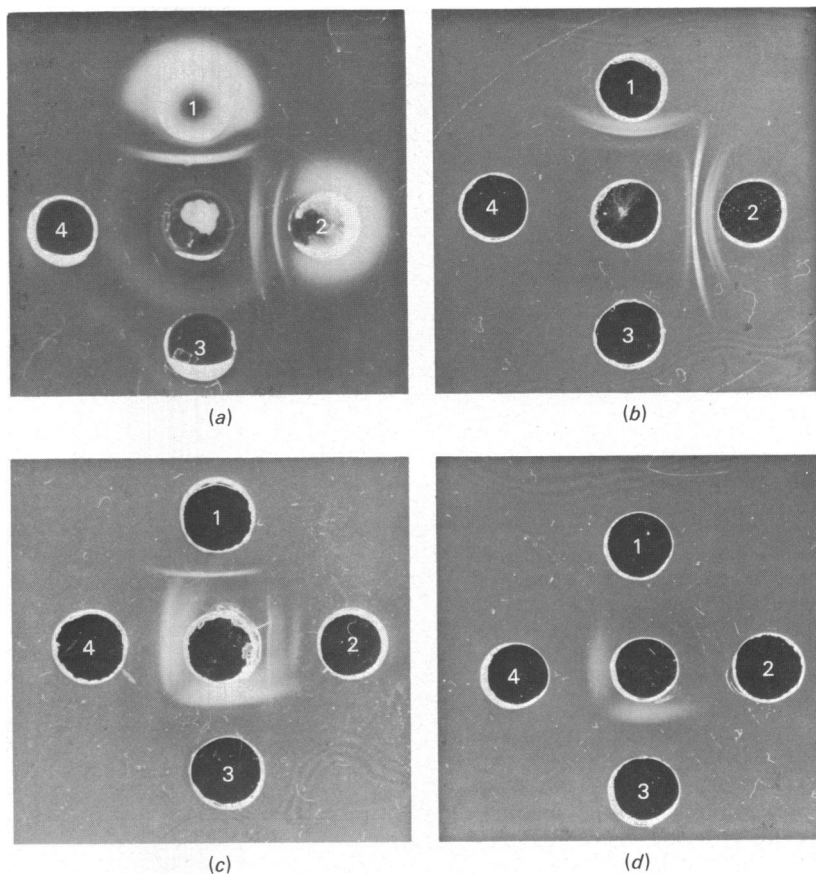


Fig. 4. Immunodiffusion of trout serum lipoproteins with specific antibodies

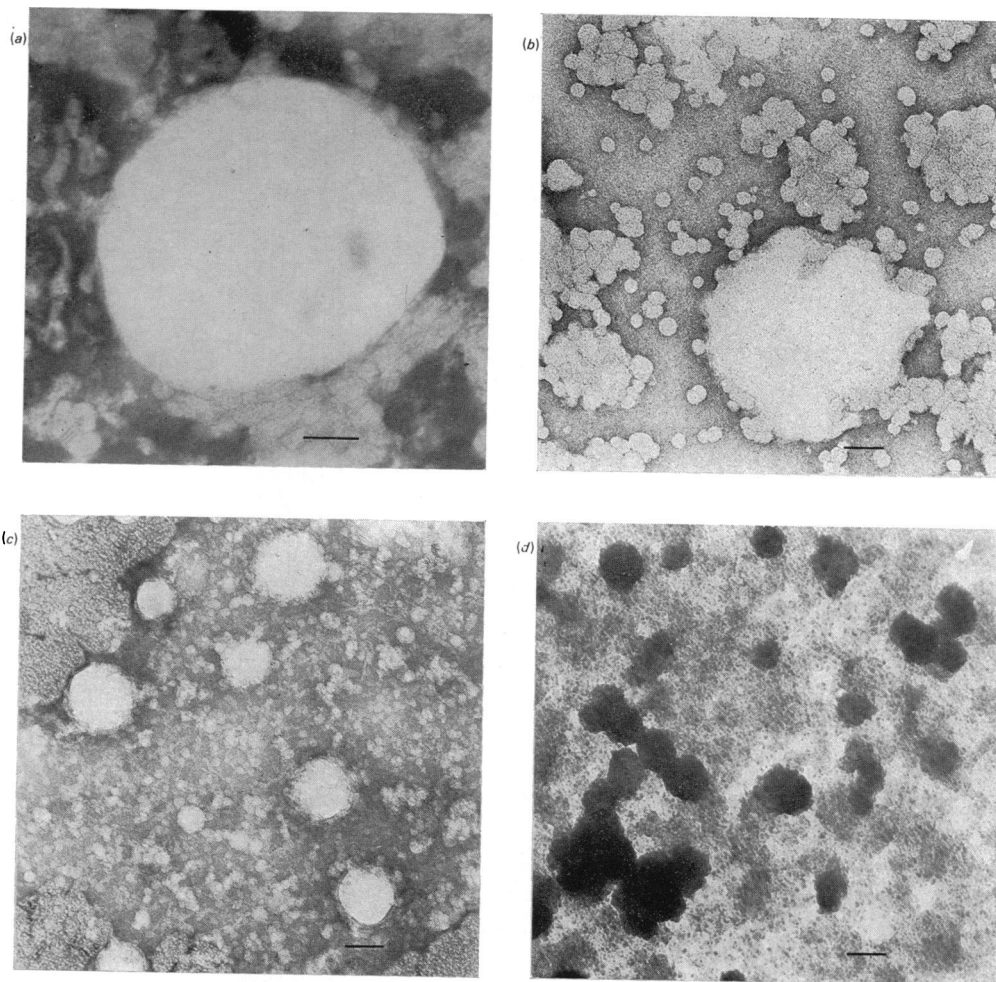
The centre wells contained (a) anti-(VLD lipoprotein), (b) anti-(LD lipoprotein), (c) anti-(HD lipoprotein+LD lipoprotein), (d) antibody used in (c) but from which the anti-(LD lipoprotein) activity had been adsorbed by the addition of LD lipoprotein. The outer wells contain trout VLD lipoprotein (1), LD lipoprotein (2) and HD lipoprotein (3 and 4).

patterns obtained (Fig. 5) were entirely consistent with those obtained by using specific antisera and confirmed that an effective separation had been achieved by centrifugation at the densities selected.

Ultracentrifugal analysis

The three lipoprotein fractions were examined in the analytical ultracentrifuge to determine their degree of homogeneity and flotation characteristics (Fig. 6). The $d < 1.020$ fraction showed a single peak of $S_0^{0.20}$ 33.7, which appeared fairly symmetrical at early times, but later displayed a broadening of the leading side, suggesting the presence of a spectrum of larger particles in an otherwise homogeneous solution. The d 1.020–1.085 fraction was also

analysed at d 1.063 as this is the conventional density at which human LD lipoproteins are analysed. As would be anticipated from the higher densities required for its isolation in the preparative ultracentrifuge (see the Materials and Methods section), this fraction had a very low rate of flotation at d 1.063, though a degree of heterogeneity was apparent. At d 1.210, however, this fraction was clearly observed to contain two components; these gave $F_{1.20}^0$ values of 18.3 and 14.7. The d 1.096–1.21 fraction on analysis at d 1.21 gave a major peak (of $F_{1.20}^0$ 3.1) that was fairly symmetrical, together with a more rapidly floating minor component. Each lipoprotein fraction was observed to be free from contamination with either of the other fractions.



EXPLANATION OF PLATE I

Trout serum lipoproteins
(a) An isolated chylomicron present in the $d < 1.020$ g/ml fraction. Scale bar represents 100 nm; (b), (c) and (d) are preparations of trout VLD, LD and HD lipoproteins respectively. Scale bar represents 100 nm in each case. All preparations were negatively stained with 2% (w/v) ammonium molybdate, pH 7.2.

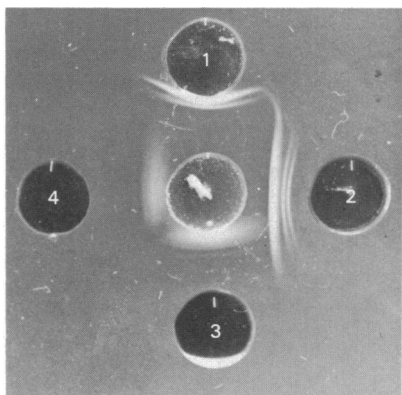


Fig. 5. Immunodiffusion of trout serum lipoproteins with anti-(trout serum)

The centre well contained rabbit antiserum raised against whole trout serum. The outer wells contained: (1) trout VLD lipoprotein; (2) trout LD lipoprotein; (3) and (4) trout HD lipoprotein.

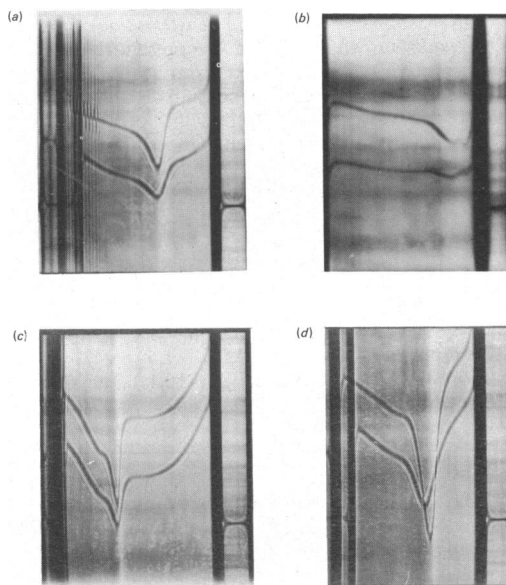


Fig. 6. Ultracentrifugal patterns of trout serum lipoproteins (a) and (b) represent the flotation profiles of VLD and LD lipoproteins respectively obtained at density 1.063 g/ml at 16 min after reaching maximum speed. (c) and (d) are the profiles of LD and HD lipoproteins respectively at density 1.210 g/ml, after 28 and 80 min respectively. The upper and lower schlieren patterns shown in each plate were obtained with the following protein concentrations: (a) 5.0 and 2.5 mg/ml; (b) and (c) 10.0 and 6.0 mg/ml; (d) 10.0 and 5.0 mg/ml. Temperature of rotor was 23.1, 23.6, 22.7 and 21.8°C for (a), (b), (c) and (d) respectively. Direction of flotation was from right to left.

Electron microscopy

The $d < 1.020$ fraction contained VLD lipoprotein-like particles of diameter 35–60 nm which were mostly spherical and symmetrical in appearance (Plate 1). Additionally, a few large particles, of diameter 300–800 nm, which displayed sharp surface features characteristic of chylomicrons, were observed along with some aggregates of VLD lipoprotein (35–60 nm) units.

The $d 1.020$ – 1.085 fraction contained spherical electron-lucent particles of diameter 10–20 nm resembling human LD lipoprotein in appearance (Forte & Nichols, 1972). Particles of diameter 120–200 nm were also present and probably represented aggregated or fused LD lipoprotein particles.

Electron-opaque particles of diameter approx. 5 nm, some of which formed aggregates of 80–120 nm diameter, were observed in the $d 1.096$ – 1.21 fraction and were characteristic of HD lipoprotein particles. No cross-contamination of particles was observed in the three fractions.

Distribution of lipoproteins

Table 1 shows the distribution of the lipoprotein fractions in serum. The agreement between the estimates obtained from scanning gels loaded with total lipoprotein and those obtained from protein determinations on the isolated fractions indicates that little loss or alteration of material occurred during the isolation procedure.

Chemical composition

The gross composition of each lipoprotein fraction is shown in Table 2. Although the greater part of

the neutral lipid of VLD lipoprotein was composed of triacylglycerol, an appreciable amount of cholesteryl ester was also present. These values were obtained from VLD lipoprotein samples prepared from the serum of fish which had been treated with oestradiol to increase the plasma VLD lipoprotein concentration, which in the normal fish is too low to permit accurate lipid analysis to be made (Skinner & Rogie, 1978). This material had the same electrophoretic and immunological properties and was composed of particles of the same size range as VLD lipoprotein isolated from untreated fish, though the existence of minor structural and compositional differences cannot be precluded. A predominant feature of LD and HD lipoproteins isolated from untreated fish is the high proportion of cholesteryl ester. For all lipoprotein fractions, only trace quantities of components other than those tabulated were observed on analysis by t.l.c.

The distribution of the major fatty acids in each lipoprotein fraction (Table 3) revealed the presence

Table 1. *Distribution of lipoproteins and lipoprotein protein in trout serum*

The results shown in column A represent the percentage distribution of lipoprotein protein calculated from the areas of respective peaks obtained from scans (at 280nm) of polyacrylamide gels after electrophoresis of the *d* 1.215 supernatant of trout serum; columns B and C give the percentage distribution and actual concentration respectively of protein, determined chemically, of each lipoprotein fraction isolated from serum by sequential flotation. The total lipoprotein contents in serum, given in column D, were calculated from the protein values of the lipoprotein fractions given in column C, by using values for the composition shown in Table 2. Determinations were made on samples of serum from two separate fish, (1) and (2).

Lipoprotein fraction	A Protein (%)		B Protein (mg/100ml of serum)		C Protein (%)		D Total lipoprotein (mg/100ml of serum)	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
VLD lipoprotein	1.5	1.5	14.5	15.3	1.3	2.8	201	212
LD lipoprotein	12.8	16.1	138.0	68.0	12.7	12.8	392	193
HD lipoprotein	85.7	82.4	935.0	448.0	86.0	84.4	2216	1062

Table 2. *Percentage composition of trout serum lipoproteins*

The values given are the means of three and five determinations on the lipoproteins prepared from separate pooled samples of trout serum of LD and HD lipoproteins respectively. The values given for the lipid components of VLD lipoprotein are the means of two preparations of lipoproteins isolated from the serum of oestradiol-treated fish (see the Materials and Methods section). The protein content of VLD lipoprotein was determined on untreated fish.

Constituent	Lipoprotein ...	VLD	LD	HD
Cholesterol		11.5	9.5	4.1
Cholesteryl ester		26.7	27.9	20.1
Triacylglycerol		38.5	12.5	5.7
Phospholipid		16.1	14.9	27.9
Protein		7.2	35.2	42.2

of large amounts of unsaturated long-chain acids in addition to the more commonly encountered palmitic acid and oleic acid.

Characterization of apoproteins

Delipidization of trout lipoproteins with ethanol/ether mixtures yielded products with differing solubility properties. Apo-(HD lipoprotein) was readily soluble in 0.9% NaCl when freshly prepared, though its solubility decreased on storage, whereas apo-(VLD lipoprotein) and apo-(LD lipoprotein) were insoluble in this solution. Apo-(VLD lipoprotein) dissolved readily in solutions containing 8M-urea, whereas apo-(LD lipoprotein) was incompletely soluble. These observations would suggest that the trout has a protein corresponding to apo-lipoprotein B, which is not very soluble in urea solutions.

Polyacrylamide-gel electrophoresis in 8M-urea of apo-(VLD lipoprotein) showed seven bands designated V-1 to V-7, of R_f 0.24, 0.36, 0.40, 0.44, 0.50, 0.66

Table 3. *Fatty acid composition of the lipid esters of trout serum lipoproteins*

Fatty acid analysis was performed by g.l.c. as described in the Materials and Methods section, and the values given represent the weight percentage of recovered fatty acids (means of two determinations on separate pooled samples; the variability of each component was normally within 1% of the total composition).

Fatty acid	Lipoprotein ...	VLD	LD	HD
C _{14:0}		1.06	0.86	1.06
C _{16:0}		15.31	17.88	21.55
C _{16:1}		6.14	4.51	4.16
C _{18:0}		4.38	4.90	3.39
C _{18:1}		30.59	26.53	19.18
C _{18:2 ω6}		5.03	4.87	4.84
C _{18:3 ω6}		0.05	0.21	0.15
C _{18:3 ω3}		0.92	0.63	0.46
C _{18:4 ω3}		1.00	0.70	0.39
C _{20:1}		3.26	2.60	1.63
C _{20:2 ω6}		0.55	0.68	0.51
C _{20:3 ω6}		1.77	0.82	0.91
C _{20:4 ω6}		1.32	1.91	2.55
C _{20:4 ω3}		2.14	1.32	0.78
C _{20:5 ω3}		3.31	4.87	5.53
C _{22:1}		0.15	0.10	0.17
C _{22:4 ω6}		2.66	0.54	0.32
C _{22:5 ω6}		1.44	2.57	1.80
C _{22:5 ω3}		2.15	1.70	1.53
C _{22:6 ω3}		16.77	21.80	29.09

and 0.86 respectively (Fig. 7). Components V-1, V-4, V-6 and V-7 were present in greatest concentrations, whereas only a trace of component V-5 was observed. Apo-(LD lipoprotein) gave one major band which migrated in the position of component V-1 (R_f 0.24) of apo-(VLD lipoprotein), though a minor component of R_f 0.1 was also present, together with an appreciable amount of aggregated material,

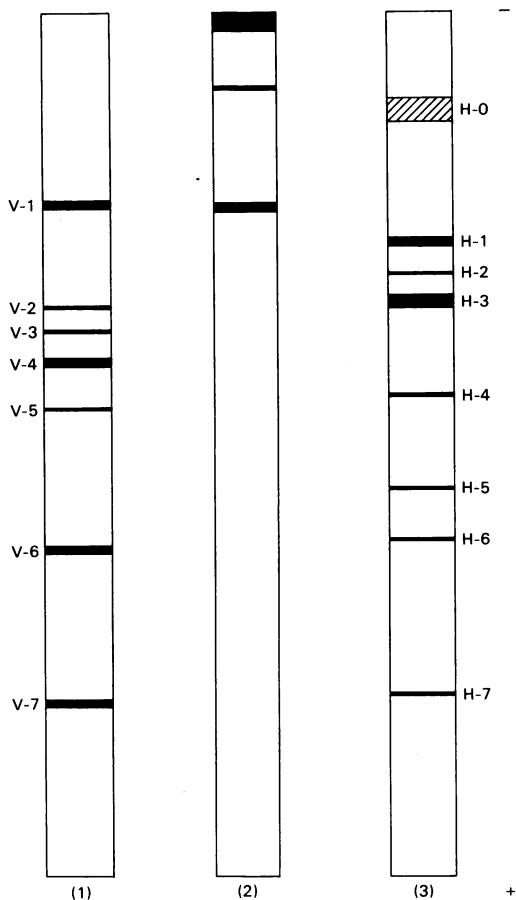


Fig. 7. Electrophoresis of trout serum apolipoproteins on polyacrylamide gels

Electrophoresis was carried out in 7.5% (w/v) acrylamide gels, without the use of spacer gels, in the presence of 8M-urea. (1) Apo-(VLD lipoprotein); (2) apo-(LD lipoprotein); (3) apo-(HD lipoprotein). R_F values are given in the text.

which remained at the origin. At least eight bands were routinely observed for apo-(HD lipoprotein); a rather diffuse band, H-0 (R_F 0.1–0.2), was shown to comprise aggregated material. Of bands H-1 to H-7, with R_F values of 0.29, 0.33, 0.36, 0.48, 0.60, 0.66 and 0.86 respectively, components H-1 and H-3 formed the main bulk of apo-(HD lipoprotein), whereas the others were present in relatively low concentrations. Additional bands of fast mobility were observed in some preparations, and may represent polymorphic forms of the components shown in Fig. 7. Components H-3, H-6 and H-7 (R_F 0.36, 0.66 and 0.86 respectively) of apo-(HD lipoprotein) thus migrated in the same positions

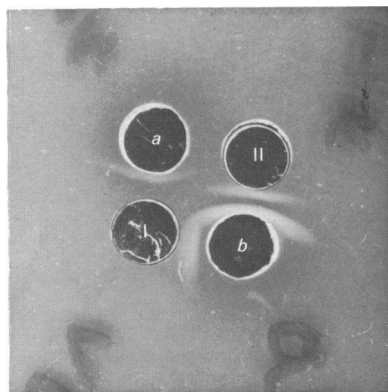


Fig. 8. Immunodiffusion of apoproteins after separation by polyacrylamide-gel electrophoresis

After electrophoresis of apo-(VLD lipoprotein) and apo-(HD lipoprotein), segments (approx. 2.0mm wide) of the polyacrylamide gels containing bands V-2 and H-3 respectively (see Fig. 7) were removed and inserted into diagonal wells cut in an agar plate (positions I and II respectively). Wells (a) and (b) were filled with anti-[trout apo-(VLD lipoprotein)] and anti-[trout apo-(HD lipoprotein)] respectively, and the plates left at approx. 20°C for 48h for the precipitin arcs to develop.

as components V-2, V-5 and V-7 of apo-(VLD lipoprotein).

The identity of band H-3 of apo-(HD lipoprotein) with band V-2 of apo-(VLD lipoprotein) was further suggested by immunodiffusion in the following manner: segments, approx. 2.0mm wide, containing these bands were cut from unstained polyacrylamide gels after electrophoresis of apo-(HD lipoprotein) and apo-(VLD lipoprotein). The location of these bands was made by a combination of scanning and R_F determinations. The two segments were inserted into preformed wells cut in an agar plate and adjacent wells filled with rabbit anti-[apo-(HD lipoprotein)] and anti-[apo-(VLD lipoprotein)]. The precipitin arcs which formed from the two segments with anti-[apo-(HD lipoprotein)] showed a clear reaction of identity (Fig. 8). The failure of the anti-[apo-(VLD lipoprotein)] to produce a similar pattern was probably a result of the weak titre of this antibody. Control discs taken from blank regions of the gel failed to produce precipitin arcs.

Gel filtration of apo-(HD lipoprotein) on Sephadex G-200 equilibrated with 0.01M-Tris/HCl buffer (pH 8.6)/8M-urea/1mM-EDTA gave an elution profile which showed three main peaks (Fig. 9). The eluted volume was, however, divided into six fractions for electrophoretic examination (Fig. 10), to facilitate identification of the bands. Fraction I, representing the first peak of the elution profile, was

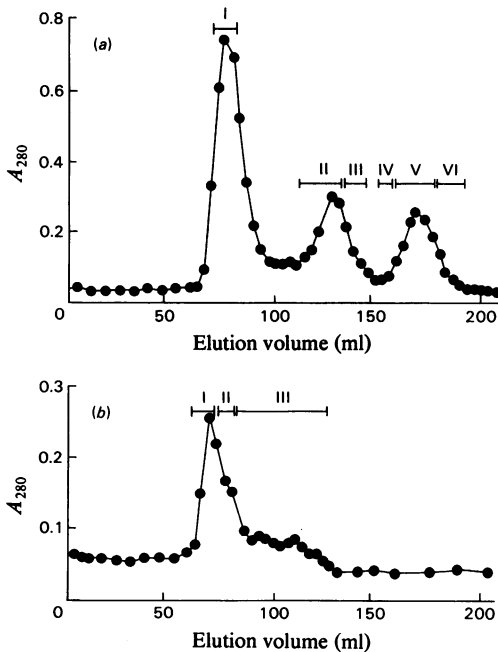


Fig. 9. Gel filtration of trout apo-(HD lipoprotein) (a) and apo-(LD lipoprotein) (b)

Approx. 30mg of apoprotein in 3ml of elution buffer was applied to a column (2.2cm \times 90cm) containing Sephadex G-200 and eluted with 0.01M-Tris/HCl (pH 8.6)/1 mM-EDTA/8M-urea as described in the Materials and Methods section. The fractions indicated by the bar lines were pooled and concentrated by ultrafiltration.

composed largely of aggregated apoproteins (band H-0), together with a small amount of H-1. Fraction II, taken from the top of the second peak, contained component H-1. Component H-6 was eluted along with H-1, in fraction III at the tail of the second peak, whereas components H-3 to H-5 were eluted in fraction V, which comprised the bulk of the third peak. The intermediate fraction (fraction IV) contained a trace of component H-1 in addition to the components in fraction V, confirming that component H-1 had been effectively resolved from the other components. Band H-7 appeared only in fraction VI.

Apo-(LD lipoprotein) was eluted from Sephadex G-200 as a single peak with some trailing material (Fig. 9b). Electrophoretic analysis (Fig. 10) revealed that the leading front of the peak (fraction I) contained the minor electrophoretic component (R_F 0.10) and aggregated material which remained at the origin. The major electrophoretic band (R_F 0.24) was confined to fraction II, taken from the following edge of the peak, where it was contaminated with the R_F 0.10

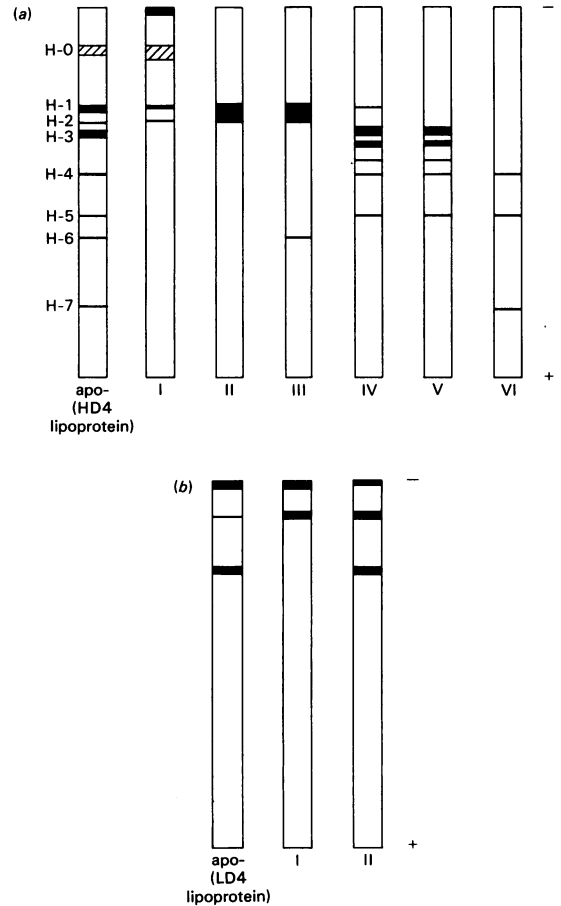


Fig. 10. Polyacrylamide-gel electrophoresis of trout apo-(HD lipoprotein) (a) and apo-(LD lipoprotein) (b) fractions obtained by gel filtration

The Sephadex G-200 fractions indicated in Fig. 9 were examined by gel electrophoresis in the presence of 8M-urea, by using the system described under Fig. 7.

component and aggregate. No electrophoretic bands were detected with material pooled from fraction III.

Insufficient apo-(VLD lipoprotein) was available to permit fractionation by gel filtration.

Amino acid analysis

The amino acid composition of trout VLD, LD and HD lipoproteins (Table 4) revealed general features, such as a high content of glutamic acid and leucine and a low content of methionine and proline, which are characteristic of the human serum apolipoproteins (see Scanu *et al.*, 1975). The three main fractions obtained by gel filtration of trout apo-(HD lipoprotein) on Sephadex G-200 (fractions I, II

Table 4. *Amino acid composition of trout serum lipoproteins*

Values are expressed as mol of each amino acid/100 mol of amino acid recovered and are the averages from three different times of hydrolysis (20, 48 and 72 h). Values for serine and threonine are corrected by extrapolation to zero time.

Amino acid	Lipoprotein ...	VLD	LD	HD
Asp		11.2	10.9	7.5
Thr		8.1	6.8	5.2
Ser		6.2	6.4	4.4
Glu		14.2	12.9	16.6
Pro		3.7	4.9	5.2
Gly		5.4	5.5	5.1
Ala		8.9	11.2	13.4
Val		5.3	6.4	7.6
Met		0	1.0	0.9
Ile		5.8	7.4	4.2
Leu		8.9	10.9	11.1
Tyr		4.1	2.4	4.8
Phe		4.5	4.2	2.1
His		1.9	1.5	1.5
Lys		8.5	5.3	6.1
Arg		3.3	2.3	4.3

and V; Fig. 9) have essentially different amino acid compositions though the contents of some residues (e.g. alanine and leucine) are very comparable (Table 5).

Discussion

In the present study, the serum lipoproteins of the trout are shown to be composed of VLD, LD and HD lipoprotein particles, which broadly resemble those of other terrestrial and aquatic species. However, the density at which trout LD lipoprotein floats in the ultracentrifuge differs from that which is conventionally used for its isolation from the serum of human and most other species. Whereas the LD and HD lipoproteins of most species are effectively separated at d 1.063, where the former floats and the latter sediments, trout LD lipoprotein consists of denser particles that completely float only at the higher density of 1.085. After centrifugation (under the conditions described) of trout serum at d 1.063, the LD lipoproteins were found, in fact, to be distributed over the upper 7.0 ml volume of the centrifuge tube, whereas at d 1.085 the HD lipoproteins remained in the lower 3.4 ml volume and completely floated only at densities greater than 1.20 g/ml (E. R. Skinner & A. Rogie, unpublished observations). In respect of its higher density, the LD lipoprotein of the trout resembles that of the cod (Skinner, 1973) and the guinea pig (Chapman *et al.*, 1975).

Each of the trout lipoprotein classes isolated by this procedure was shown to be free from con-

Table 5. *Amino acid composition of apoproteins of trout HD lipoprotein*

Fractions I, II and V refer to those obtained by gel filtration on Sephadex G-200 in the presence of 8M-urea as described in the text and indicated in Fig. 9. Values are expressed as mol of each amino acid/100 mol of amino acid recovered and are the averages from three different times of hydrolysis (20, 48 and 72 h), those for serine and threonine being corrected by extrapolation to zero time. The data for salmon DEAE-cellulose fraction 3 are from Nelson & Shore (1974) and represent the composition of the DEAE-cellulose fraction 3 of salmon HD lipoprotein which may correspond to human apoprotein A-I. The values for human apoprotein A-I are calculated from the primary structure.

Amino acid	Salmon; DEAE-cellulose; Fraction 3			Human apoprotein A-I	
	I	II	V		
Asp	10.6	5.4	7.6	5.8	8.7
Thr	6.3	4.3	3.3	5.3	4.2
Ser	7.6	4.1	3.6	4.9	5.8
Glu	14.1	18.1	13.2	19.9	19.5
Pro	3.6	4.8	6.8	3.8	4.1
Gly	6.7	2.6	8.8	2.1	4.1
Ala	12.5	13.2	12.4	12.0	7.9
Val	8.3	6.7	9.5	6.5	5.4
Met	0	2.4	1.8	3.3	1.3
Ile	6.0	4.5	3.8	3.7	0
Leu	10.1	10.3	9.1	11.0	16.2
Tyr	0	3.7	5.9	4.0	2.9
Phe	0	2.3	2.2	1.6	2.5
His	1.7	1.2	1.6	1.6	2.1
Lys	8.4	9.9	8.1	9.8	8.7
Arg	4.1	6.5	2.3	4.7	6.6

tamination by other lipoprotein classes as judged by electrophoresis, immunodiffusion, analytical ultracentrifugation and electron microscopy. This has enabled a detailed comparison to be made between the trout lipoproteins and their human counterparts and also to establish the interrelations that exist between the different trout lipoprotein classes.

The lipid composition of dogfish (Lauter *et al.*, 1968) and sardine (Lee & Puppione, 1972) lipoproteins have been determined and, in addition, the apoprotein composition of the shark, *Centrophorus squamosus*, has been investigated (Mills *et al.*, 1977). In these investigations lipoprotein fractions, isolated by flotation at densities that are conventionally used for the preparation of human lipoproteins, were shown to contain multiple apoprotein bands on electrophoresis in urea and some of the components were partially characterized. Although the HD lipoprotein of cod (Skinner, 1973) and pre-spawning salmon (Nelson & Shore, 1974) have been characterized with respect to both their lipid and apoprotein constituents, no information has hitherto

been available on the composition and inter-relationship of all three major lipoprotein classes of any fish species.

The VLD lipoprotein fraction of trout serum, isolated at d 1.020, was shown by electron microscopy to contain a small number of chylomicrons in addition to the VLD lipoprotein particles themselves. Both components showed a marked similarity in appearance and size to those reported for the corresponding human components (Forte & Nichols, 1972). Analysis of this fraction in the analytical ultracentrifuge gave a possible indication of the presence of chylomicrons in low concentration during acceleration of the rotor, whereas the VLD lipoprotein pattern suggested a fairly symmetrical distribution of particle size. The S_0^0 value of 33.7 was in the upper regions of the range (S_0^0 20–40) reported for human VLD lipoprotein (Scanu, 1972), suggesting that trout VLD lipoprotein particles are rather larger than those present in most human subjects. Chylomicrons and VLD lipoprotein separated on polyacrylamide gels in a manner characteristic of their human counterparts (Frings *et al.*, 1971) and their immunological identity showed that, as with the human (Kostner & Holasek, 1972), they have apoproteins in common.

Trout LD lipoprotein consists of particles that are smaller and denser than those isolated from human serum. They varied in diameter from 10 to 20 nm, compared with 17–26 nm for human LD lipoprotein (Forte & Nichols, 1972), and in the analytical ultracentrifuge resolved into two components of $F_{1,20}^0$ 18.3 and 14.7. Although the pattern resembles that reported for normal human subjects, the $F_{1,20}^0$ values were lower than that of the human (Nelson & Morris, 1977). The presence of two major components was confirmed by analysis on polyacrylamide gels, which revealed an additional minor component. The behaviour of the two major components on polyacrylamide-gel electrophoresis and the observation that they contain common antigenic determinants is analogous to that of human lipoproteins LD 1 and LD 2, both of which contain apolipoprotein B (Alaupovic *et al.*, 1972).

The HD lipoprotein of trout serum appeared as uniform particles of approx. 5 nm diameter in the electron microscope, and in the analytical ultracentrifuge gave a major peak that was fairly symmetrical, together with a second minor component. However, a combination of electrophoretic and immunological methods revealed the presence of three sub-species, resembling the situation in the human (Alaupovic *et al.*, 1972); the major components appear to correspond to human HD lipoproteins 2 and 3. The proportions of these two components were found to vary in different preparations and in different samples of fresh serum, suggesting that they may be present in this state in the

fish and are not artifacts produced during the isolation procedure.

The distribution of lipoprotein classes varies enormously in different species and is probably a reflection of different rates of metabolic activity. This is particularly noticeable in fish, where in *Centrophorus* VLD lipoprotein forms the major constituent (Mills *et al.*, 1977), whereas prespawning salmon contain only HD lipoprotein (Nelson & Shore, 1974). The trout provides a good model for lipoprotein investigations, since significant amounts of all three lipoprotein classes are present, although HD lipoprotein is the dominant species.

The metabolic processes associated with the transport and deposition of lipids in the human and rat are now fairly well understood (Eisenberg & Levy, 1975) and it is of interest to know whether similar processes occur in fish. Inferences may be obtained from a knowledge of the distribution of apoproteins between the different lipoprotein classes.

The patterns obtained on electrophoresis in 8M-urea of the delipidized apolipoproteins show a resemblance to those obtained with the human (Scanu, 1972). Thus the slowest of the seven bands produced by trout apo-(VLD lipoprotein) corresponds to the main band of apo-(LD lipoprotein). This component is absent from apo-(HD lipoprotein) and therefore appears to correspond to apoprotein B of the human. The major components of trout apo-(HD lipoprotein) (i.e. H-1 and H-3) migrate in approximately the same positions as apoproteins A-I and A-II of human HD lipoprotein and also show gel-filtration characteristics similar to those of human apoproteins A-I and A-II respectively (Scanu *et al.*, 1969). Clearly a proper assignment of these components must await detailed analysis of the isolated apoproteins, but it is noteworthy that apoprotein H-3 of trout apo-(HD lipoprotein) migrates in the same position as apoprotein V-2 of trout apo-(VLD lipoprotein) and when sections of polyacrylamide gels containing these bands reacted with anti-[apo-(HD lipoprotein)] a reaction of identity was obtained. Some of the faster-migrating components of trout apo-(HD lipoprotein) which were eluted in fractions V and VI on gel filtration and correspond in some cases to components present in apo-(VLD lipoprotein) may represent counterparts to the human C apolipoproteins.

The amino acid composition of fraction II of trout apo-(HD lipoprotein), which contains essentially pure apoprotein H-1 (Fig. 10), is remarkably similar to that reported by Nelson & Shore (1974) for one of the major apoproteins of salmon HD lipoproteins which exhibits the same electrophoretic characteristics as trout apoprotein H-1. These authors showed that salmon apo-(HD lipoprotein) was resolved on DEAE-cellulose columns into six fractions, of which fractions 3 and 4 migrated in the

same position on polyacrylamide-gel electrophoresis and presumably represent polymorphic forms of the apoprotein. Trout apoprotein H-1 therefore appears to correspond to the DEAE-cellulose fraction 3 of salmon apo-(HD lipoprotein) and to apoprotein A-I of human serum. Both the trout and the salmon apoproteins contain considerably smaller amounts of aspartic acid and leucine than does human apoprotein A-I, but both have appreciably greater contents of alanine and isoleucine (see Table 5).

Trout VLD, LD and HD lipoproteins each contain multiple antigenic determinants. At least one of these is common to VLD and LD lipoproteins. However, reactions of non-identity were shown between the precipitin lines produced by HD and LD lipoproteins, and adsorption of antisera with LD lipoprotein had no effect on HD lipoprotein though eliminating reactivity with LD lipoprotein. These observations suggest that LD and HD lipoproteins contain separate and distinct sets of apoproteins, some of which in each case are also present in VLD lipoprotein. These relationships show a close parallel to those in the human. This would imply that the individual apoproteins of the trout serve structural and functional roles similar to those of the corresponding human components. Thus trout apoprotein V-1, like human apoprotein B, may form part of the major structural subunit of chylomicrons, VLD lipoprotein and LD lipoprotein, whereas trout apoprotein H-3 may form the structural subunit of HD lipoprotein, as does apoprotein A-II in the human. The low-molecular-weight apoproteins present in trout VLD and HD lipoproteins, which appear to correspond to the human C peptides, may serve a regulatory role.

The close resemblance between the lipoproteins of the trout and human suggests that the serum lipoproteins were evolved early in evolutionary history and that they have been preserved throughout the development of species in a broadly similar form. This finding is in agreement with the common-ancestry hypothesis based on a comparison of the sequences of four human apoproteins (Barker & Dayhoff, 1977). Although the observed differences in the amino acid composition of the individual peptides are to be expected as a result of a series of single point mutations, it may be assumed that the modified proteins have retained their functional specificity. These structures are presumably essential for any organism which depends on lipid as a source of energy. It has been shown by Mills & Tylaur (1971) that members of the reptile and bird classes contain lipoproteins of the same density ranges and which are composed of the same lipid components. Two species of birds are reported to have broadly similar apolipoprotein patterns (Hearn & Bensadoun, 1975; Kelly & Alaupovic, 1976). Although other teleost fish (Skinner, 1973; Nelson &

Shore, 1974) possess structures that are very similar to those of the trout, a report by Mills *et al.* (1977) on the lipoproteins of *Centrophorus squamosus* indicates that at least some of the components found in higher animals are also present in the evolutionarily simpler elasmobranchs. Species of fish, such as trout, may therefore provide useful models for the study of certain serum lipoproteins.

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References

- Alaupovic, P., Lee, D. M. & McConathy, W. J. (1972) *Biochim. Biophys. Acta* **260**, 689–707
- Allen, C. F., Good, P., Davis, H. F., Chisum, P. & Fowler, S. D. (1966) *J. Am. Oil Chem. Soc.* **43**, 223–231
- Barker, W. C. & Dayhoff, M. O. (1977) *Comp. Biochem. Physiol.* **B57**, 309–315
- Bersot, T. P., Brown, W. W., Levy, R. I., Windmueller, H. G., Fredrickson, D. S. & LeQuire, V. S. (1970) *Biochemistry* **9**, 3427–3433
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Bodman, J. (1960) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), vol. 2, pp. 91–157, William Heinemann, London
- Chapman, M. J., Mills, G. L. & Ledford, J. H. (1975) *Biochem. J.* **149**, 423–436
- Chapman, M. J., Goldstein, S., Mills, G. L., Fremont, L. & Leger, C. (1978) *Protides Biol. Fluids Proc. Colloq.* **25**, 487–490
- Cowey, C. B., Owen, J. M., Adron, J. W. & Middleton, C. (1976) *Br. J. Nutr.* **36**, 479–486
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Eisenberg, S. & Levy, R. I. (1975) *Adv. Lipid Res.* **13**, 1–89
- Forte, T. & Nichols, A. V. (1972) *Adv. Lipid Res.* **10**, 1–41
- Frings, C. S., Foster, L. B. & Cohen, P. S. (1971) *Clin. Chem.* **17**, 111–114
- Gustafson, A., Alaupovic, P. & Furman, R. H. (1965) *Biochemistry* **4**, 596–605
- Hearn, V. & Bensadoun, A. (1975) *Int. J. Biochem.* **6**, 295–301
- Hornstein, I., Crowe, P. F. & Ruck, J. B. (1967) *Anal. Chem.* **39**, 352–354
- Kelly, J. L. & Alaupovic, P. (1976) *Atherosclerosis* **24**, 155–176

- Koga, S., Horowitz, D. L. & Scanu, A. M. (1969) *J. Lipid Res.* **10**, 577-588
- Koga, S., Bollis, L. & Scanu, A. M. (1971) *Biochim. Biophys. Acta* **236**, 416-430
- Kostner, G. & Holasek, A. (1972) *Biochemistry* **11**, 1217-1223
- Lauter, C. J., Brown, E. A. B. & Trams, E. G. (1968) *Comp. Biochem. Physiol.* **24**, 243-247
- Lee, R. F. & Puppione, D. L. (1972) *Biochim. Biophys. Acta* **270**, 272-278
- Lindgren, F. T., Jensen, L. C. & Hatch, F. T. (1972) in *Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism* (Nelson, G. J., ed.), pp. 181-274, John Wiley, New York
- Lis, E. W., Tinoco, J. & Okey, R. (1961) *Anal. Biochem.* **2**, 100-106
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Miller, G. L. (1959) *Anal. Chem.* **31**, 964
- Mills, G. L. & Taylaur, C. E. (1971) *Comp. Biochem. Physiol.* **B40**, 489-501
- Mills, G. L., Taylaur, C. E., Chapman, M. J. & Forster, G. R. (1977) *Biochem. J.* **163**, 455-465
- Nelson, C. A. & Morris, M. D. (1977) *Biochem. Med.* **18**, 1-9
- Nelson, G. J. & Shore, V. G. (1974) *J. Biol. Chem.* **249**, 536-542
- Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand.* **26**, 507-515
- Scanu, A. M. (1972) *Biochim. Biophys. Acta* **265**, 471-508
- Scanu, A. M. & Edelstein, C. (1971) *Anal. Biochem.* **44**, 576-588
- Scanu, A., Toth, J., Edelstein, C., Koga, S. & Stiller, E. (1969) *Biochemistry* **8**, 3309-3316
- Scanu, A. M., Edelstein, C. & Keim, P. (1975) in *The Plasma Proteins* (Putnam, F. W., ed.), vol. 1, 2nd edn., pp. 317-391, Academic Press, New York
- Skinner, E. R. (1973) *Biochem. Soc. Trans.* **1**, 434-436
- Skinner, E. R. & Rogie, A. (1978) *Protides Biol. Fluids, Proc. Colloq.* **25**, 491-494