Characterization of Serum Lipoproteins of the Shark *Centrophorus squamosus*

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1. Blood serum from the shark *Centrophorus squamosus* (Bonnaterre) was shown to contain VLD (very-low-density), LD (low-density) and HD (high-density) lipoproteins. 2. In shape, size and general physical properties, these lipoproteins were very similar to those described for other animals. The VLD lipoproteins were the major components of the mixture, and HD lipoproteins were present at the lowest amount. 3. In addition to the usual lipid components, the shark lipoproteins also contain substantial amounts of hydrocarbon, probably mainly squalene, and monoalkyldiacylglycerols. Only trace amounts of wax ester were detected. 4. The protein moiety of the VLD and LD lipoproteins contained a component which, in its solubility and electrophoretic properties, molecular weight and amino acid composition, resembled the B apolipoprotein of man and other mammals. This accounted for a large part of the total shark apolipoprotein. 5. There were also present smaller amounts of proteins which were soluble in 8M-urea. In their electrophoretic mobility on basic polyacrylamide gel, some of these were like the A and C apoproteins of man. 6. The electrophoretic distribution of the soluble proteins from the VLD and LD lipoproteins resembled that in higher mammals, but in the HD lipoproteins the similarity was less.

The study of the serum lipoproteins of fish has not progressed so rapidly, nor so far, as the investigation of mammalian lipoproteins. Nonetheless, there is evidence that the same lipids are present in the lipoproteins from animals of both these classes, albeit not always in the same proportions (Lauter *et al.*, 1968; Mills & Taylaur, 1971; Lee & Puppione, 1972; Nelson & Shore, 1974). However, fish tissues commonly contain large amounts of very-long-chain highly unsaturated fatty acids, and in sharks may also include appreciable amounts of hydrocarbons and alkyldiacylglycerols (Heller *et al.*, 1957; Hallgren & Larsson, 1962; Lambertson & Holman, 1963; Malins *et al.*, 1965; Bone & Roberts, 1969; Malins & Wekell, 1971). Sargent *et al.* (1973) have also shown that both hydrocarbons and alkyldiacylglycerols are present in the serum lipoproteins of certain sharks. A detailed comparative study of the lipoproteins of fish and mammals should therefore help to determine the structural changes that result from the presence of the unusual components in fish, and may also clarify their essential structure. It should also contribute to an understanding of the evolution of serum lipoproteins as the agent of fat transport. It is with these objects in view that the following study of the serum lipoproteins of *Centrophorus squamosus* (Bonnaterre) is reported.

Experimental

Materials

The reagents and solvents used were of analytical grade, purchased from BDH, Poole, Dorset, U.K. In addition, Bio-Sil HA (–325 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., and silica gel G from E. Merck, Darmstadt, Germany. Reference mixtures of fatty acids were obtained from Supelco Inc., Bellefonte, PA, U.S.A., and authentic α -(octadecyloxy)-1,2-propanediol (batyl alcohol) and α -(hexadecyloxy)-1,2-propanediol (chimyl alcohol), squalene and cetyl palmitate from Sigma Chemical Co., Kingston-on-Thames, Surrey, U.K. A mixture of alkyldiacylglycerols from dogfish liver was given by Dr. J. R. Sargent, Institute of Marine Biochemistry, Aberdeen, Scotland, U.K.

Fish

A total of eight *Centrophorus squamosus* (Bonnaterre) was caught at depths of 1000–1150 m in the Bay of Biscay. The fish were moribund when brought inboard but were still making weak tail movements. They were suspended tail downwards and blood samples immediately taken from the caudal vein into precooled tubes, where it was allowed to clot for 20–30 min at about 5°C. The serum was then pipetted

off and deep-frozen, and 3 days later the frozen serum was sent by rail from Cardiff to London, where analyses were begun at once. Serum which was not required immediately was kept at -16°C .

Methods

Isolation of serum lipoproteins. Lipoproteins were isolated from serum by sequential ultracentrifugation at 12°C . All the solutions used contained 1 mM-EDTA and 0.01% (w/v) NaN_3 , and their densities were determined by pycnometry.

Since the density of the protein-free solvent phase of shark serum is about 1.025 g/ml (Sargent *et al.*, 1973), the conventional lipoprotein subfractions could only be obtained by a modification of the usual technique. For the preparation of VLD lipoproteins* (i.e. of hydrated density less than 1.007 g/ml), 4.5 ml of native serum was overlaid with 4.5 ml of NaCl solution of density 1.007 g/ml, and centrifuged for 16 h in the Spinco 30.2 rotor (30000 rev./min; 79420g). The VLD lipoproteins were then aspirated from the top of the tube in 2 ml. The remaining 7 ml of diluted serum was mixed with 2 ml of NaCl solution of density 1.2275 g/ml, and the centrifugation repeated under the same conditions. The lipoproteins of density 1.007–1.065 g/ml (LD lipoproteins) were then aspirated from the top of the tube in a volume of 1 ml. In a later modification of the procedure, the second adjustment of the density was carried out by dialysis against NaCl solution of density 1.063 g/ml. After removal of the lipoproteins, a further 2 ml of solution was also removed and discarded. The remainder was mixed with an equal volume of NaBr solution (density 1.357 g/ml) and centrifuged for 48 h in the Spinco 40.3 rotor (40000 rev./min; 114500g). The HD lipoproteins (density 1.065–1.21 g/ml) were also recovered by aspiration.

The three lipoprotein fractions were each washed once by centrifugation in salt solutions of density 1.007, 1.063 and 1.21 g/ml respectively.

* Abbreviations: VLD lipoprotein, very-low-density lipoprotein; LD lipoprotein, low-density lipoprotein; HD lipoprotein, high-density lipoprotein.

For examination by analytical ultracentrifugation, lipoproteins were isolated essentially as described by DeLalla & Gofman (1954), with the following modifications to accommodate the high solvent density of the fish serum. The low-density compounds ($d < 1.063$) were obtained by centrifuging a mixture of 1 vol. of serum with 2 vol. of NaCl of density 1.082 g/ml for 18 h at 12°C in the Spinco 30.2 rotor (30000 rev./min; 79420g). After removal of the supernatant layer of lipoproteins in a volume of 1 ml, a further 2 ml was removed and discarded. The remainder was then mixed with an equal volume of NaBr solution (density 1.357 g/ml) and centrifuged in the Spinco 40.3 rotor for 48 h at 12°C (40000 rev./min; 114500g). The supernatant HD lipoproteins were also collected in 1 ml.

Separation of lipids and proteins. Approx. 12 mg of lipoprotein (in about 1 ml of solution) was extracted with 25 ml of ethanol/ether (3:1, v/v) at room temperature (15 – 28°C) for 16–24 h. The protein was centrifuged down at 3000g for 30 min and, after removal of the supernatant, was washed once more with the same solvent under the same conditions. Samples of the extract were evaporated to dryness under N_2 at about 50°C and a preliminary separation of the lipid components was done by chromatography on silicic acid by using a modification of the method of Hirsch & Ahrens (1958). Approx. 3 mg of extract, dissolved in light petroleum (b.p. 40 – 60°C) was applied to 1 g of Bio-Sil HA in a column ($9\text{ cm} \times 0.7\text{ cm}$). The individual lipids were then eluted by the sequence of solvents given in Table 1.

T.l.c. on plates of silica gel G $250\mu\text{m}$ thick was used to monitor the purity of these fractions, and to separate the mixtures which were not resolved on the column. After development in the appropriate solvent, the t.l.c. plates were sprayed with 0.02% dichlorofluorescein in 95% (v/v) ethanol and inspected under u.v. light. The required areas were scraped off the plate, and the lipid was eluted with diethyl ether.

The triacylglycerols and the alkyldiacylglycerols which formed fraction III from the columns were

Table 1. *Mixtures of lipids extracted from isolated lipoproteins as described in the text eluted separately from columns of Bio-Sil HA by this sequence of solvents*

The boiling point of the light petroleum used was 40 – 60°C .

Fraction	Lipid	Solvent	Volume (ml)
I	Hydrocarbons	5% Benzene in light petroleum	100
II	Cholesterol esters and waxes	40% Benzene in light petroleum	20
III	Triacylglycerols	80% Benzene in light petroleum	100
	Alkyldiacylglycerols		
IV	Cholesterol	100% Benzene	50
V	Diacylglycerols	Chloroform	50
	Monoacylglycerols		
VI	Phospholipids	Methanol/chloroform/water (87:10:3, by vol.)	40

resolved by t.l.c. in light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (90:10:1, by vol.), and this system was also used to establish the purity of fraction I. The mono- and di-acylglycerols of fraction V could be resolved by t.l.c. in light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (80:20:1, by vol.), and the same system was used to check the purity of fraction IV.

According to Nicolaides *et al.* (1970), cholesterol esters may be separated from wax esters by t.l.c. in *n*-hexane/diethyl ether (19:1, v/v). However, the cholesterol esters of fraction II from *Centrophorus* lipids were themselves resolved by this system into two subfractions, referred to as CE1 and CE2. Of these, the faster-running (CE1) contained the cholesterol esterified with fatty acids of chain length up to arachidonic acid, whereas the esters in the slower spot were predominantly those of the highly unsaturated acids of longer chain length. Because these substances (CE2) occupied the position where wax esters would be expected to appear, the following procedure was used in an attempt to determine whether waxes were present. The CE2 fractions from several t.l.c. plates were combined, hydrolysed by the method of Albrink (1959), and the liberated cholesterol and fatty acids determined by the methods referred to below. Since the amount of cholesterol was almost equivalent to the fatty acid, it was evident that the proportion of wax present must be very small (cf. the Results section).

Determination of lipids and protein. The chemical determination of cholesterol, phospholipid, fatty acids and protein was carried out as described by Mills *et al.* (1972) and by Mills & Taylaur (1971, 1973). Hydrocarbon and alkyldiacylglycerol were determined gravimetrically after the appropriate areas from t.l.c. plates had been eluted with diethyl ether. These eluates were filtered through a sintered-glass funnel, evaporated under a stream of N₂ at 50°C, and dried *in vacuo* to constant weight. Corresponding areas from blank sections of the same plate were extracted and weighed in the same way.

Factors for converting ester cholesterol into the equivalent weight of cholesterol ester, and the concentration of glyceride fatty acids into the equivalent weight of glyceride, were obtained by calculating the mean molecular weight of the relevant fatty acid mixture from its g.l.c. analysis. In this way the factor 1.67 was derived for fraction CE1, and factors of 297.6, 312.9 and 358.9 were found for tri-, di- and mono-acylglycerol respectively.

Gas-liquid chromatography. Samples of fatty acids liberated from the lipid esters by saponification (Albrink, 1959) were converted into their methyl esters with diazomethane. The glycerol monoethers produced by the saponification of alkyldiacylglycerols were converted into their isopropylidene derivatives as described by Malins *et al.* (1965). Both

the fatty acid esters and the isopropylidene derivatives were analysed on a Perkin-Elmer F11 gas chromatograph, equipped with a 180 cm × 0.64 cm (6 ft × 0.25 in) stainless-steel column packed with 20% diethylene glycol succinate polymer on Chromosorb W (80–100 mesh). The column temperature was 175°C and the carrier gas N₂. The resolved components were identified by comparison with the esters of standard fatty acid mixtures, or by reference to authentic chimyl and batyl alcohols. The relative amounts of the components were determined from the product of peak height and retention time (Carroll, 1961).

The hydrocarbon isolated from the lipoproteins was analysed by g.l.c. under the same conditions as those used for the fatty acid esters, and also on a column of SE30 (2.5% on Chromosorb AW-DMDC, 60–80 mesh) temperature-programmed from 170 to 260°C at 1.5°C/min.

Amino acid analysis. Portions (2–5 mg) of the dried protein obtained by exhaustive ethanol/ether extraction of the lipoprotein preparations were hydrolysed in 6M-HCl for 22 h at 110°C as described by Chapman *et al.* (1975). Amino acid analyses were then performed by the procedure of Moore *et al.* (1958) on a JEOL JLC5AH instrument (Japan Electron Optics Laboratory Co., Tokyo, Japan). Neither tryptophan nor cystine was determined.

Gel-filtration chromatography. The procedure for the fractionation of the apo-LD lipoproteins was based on that used by Herbert *et al.* (1974) for the fractionation of human apo-VLD lipoproteins.

Columns of acrylic tube (Pharmacia, Uppsala, Sweden; 60 × 0.9 cm) were filled with Sephadex G-200 which had previously been equilibrated with the eluting buffer: 0.01 M-Tris/HCl at pH 8.0, containing 2 mM-sodium decyl sulphate, 3 mM-NaN₃ and 0.001% (w/v) merthiolate. Up to 5 mg of apo-LD lipoprotein, in a volume of not more than 1 ml, could be applied to these columns, which were run at 20°C and at flow rates of 10–18 ml/h. The effluent was monitored continuously at 280 nm on a Gilson Spectrochrom MD coupled to an Altex recorder (Linear Instrument Corp., Palo Alto, CA, U.S.A.), against a reference cuvette containing the eluting buffer. Fractions (1–2 ml) were collected in a Gilson Microcol TDL 80 fraction collector.

Peak fractions were normally pooled and freeze-dried immediately after the column was completely eluted. The proteins were then dissolved by addition of a small volume of water to give a final concentration of sodium decyl sulphate in the range 5–15 mM. Complete and ready solution of the material which was eluted at the void volume (fraction I) was usually achieved by this procedure. Recovery of protein from the columns exceeded 85%, as judged by the assay method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Electrophoresis. Electrophoresis in the sodium dodecyl sulphate / polyacrylamide - gel system of Weber & Osborn (1969) was performed as described by Chapman *et al.* (1975).

The apoprotein fraction that could be extracted into 0.005 M-Tris/HCl buffer, pH 8.0, containing 8 M-urea, was analysed in 7.5% (w/v) polyacrylamide gels containing 8 M-urea, essentially by the modification of the procedure of Davis (1964) described by Reisfeld & Small (1966). Samples containing approx. 100 µg were applied to each gel. The conditions of running and staining were as described by Chapman *et al.* (1975). Bromophenol Blue was used as marker dye, and the current was passed until the dye front was within 1 cm of the bottom of the gel. The electrophoretic mobility of the separated peptides was expressed as the ratio of the distance of migration to that of the dye front.

Electron microscopy. LD lipoproteins were negatively stained by a procedure based on that of Forte *et al.* (1968). The lipoproteins were diluted with double-distilled water to a final concentration of 0.1–0.3 mg of protein/ml before mixing with an equal volume of 1% potassium phosphotungstate solution, pH 7.4. A drop of this mixture was applied to a Parlodion-coated carbon-sprayed copper grid; the excess was removed and the grid allowed to dry. These preparations were examined at 60 kV in a Phillips EM300 electron microscope equipped with an anti-contamination device. The sizes of isolated clearly defined particles were measured directly from the negatives with a micro-comparator graduated to 0.05 mm (L'Optique Scientifique, Paris, France). Not less than 200 particles were measured, and the microscope was calibrated by observations on a germanium-shadowed carbon replica of a ruled diffraction grating bearing 54 864 lines/in (21 600 lines/cm).

Analytical ultracentrifugation. The distribution of lipoproteins in fractions isolated at 1.063 g/ml and

1.21 g/ml was determined in a Spinco model E ultracentrifuge at 52 640 rev./min and 26°C, essentially as described by DeLalla & Gofman (1954). The coordinates of the schlieren photographs were transferred on to punched paper tape by means of a P.C.D. digital data reader (P.C.D. Ltd., Farnborough, Hants., U.K.) and subsequently processed by a digital computer according to a program similar to that described by Ewing *et al.* (1965). This presented the LD lipoprotein profile in the four segments which are conventionally described as S_r 0–12, S_r 12–20, S_r 20–100 and S_r 100–400 (DeLalla & Gofman, 1954). Each segment includes all the lipoprotein particles of flotation rate (S_r) between the quoted limits, which are expressed in Svedberg units. The amount of lipoprotein in each fraction was given in mg/100 ml of serum. The reproducibility of the analysis had been determined in earlier experiments and expressed as the technical error, defined as $\sqrt{\frac{\sum d^2}{2N}}$, where d is the difference between duplicate estimations, and N the number of duplicates. The values obtained (in mg/100 ml) for S_r 0–12, S_r 12–20, S_r 20–100 and S_r 100–400 were ± 11.3 , ± 4.9 , ± 7.3 and ± 16.8 respectively.

Results

Although serum was obtained from several fish, it was not possible to complete all the analyses on a single sample. The specimens were therefore mixed into a single pool.

Distribution and gross composition of Centrophorus lipoproteins

The distribution of the serum lipoproteins of *C. squamosus* is expressed in the conventional form (DeLalla & Gofman, 1954) in Table 2, where it is compared with data for other fish and reptiles. Both LD and HD lipoproteins were present, with the sub-

Table 2. *Distribution of serum lipoproteins from C. squamosus*

Comparable data are given for other members of the classes Pisces, Amphibia and Reptilia (Mills & Taylaur, 1971, 1973). Each component is quoted in mg of lipoprotein/dl of serum determined from a single analysis of a pool of serum; the technical errors of the determinations are given under 'Methods'. The modal S_r (flotation) rate is defined under 'Methods' and is expressed in Svedberg units. N.D., Not determined.

	HD lipoprotein	LD lipoprotein fractions				Modal S_r
		S_r 0–12	S_r 12–20	S_r 20–100	S_r 100–400	
<i>Centrophorus squamosus</i>	40	185	45	256	159	6.1
<i>Latimeria chalumnae</i>	127	74	120	875	230	10.9
<i>Myxine glutinosa</i>	553	489	221	1074	593	3.8
<i>Scyliorhinus canicula</i>	23	136	18	28	0	5.9
<i>Conger vulgaris</i>	N.D.	189	36	228	228	4.6
<i>Rana temporaria</i>	29	55	38	99	0	6.1
<i>Natrix natrix</i>	382	359	48	58	149	4.1

stances of S_r 20–400 (VLD lipoproteins) making the greatest contribution on a weight basis. The amount of S_r 0–20 (LD) lipoproteins, although smaller, was still substantial, but the HD lipoproteins were present only at a very low concentration.

By electron microscopy, the VLD and LD lipoproteins appeared to be of approximately circular profile, although deformed in a crowded field. HD lipoproteins were not available in sufficient amount for study by this technique. The VLD lipoprotein fraction was extremely heterogeneous, but the LD lipoproteins ($d=1.006$ – 1.063) had a well-defined mean diameter of 23.8 nm, with an extreme range of 17.5 to 30.0 nm and a standard deviation of ± 3.2 nm. No evidence of subunit structure could be seen in these preparations.

The percentage composition by weight of the three main classes of lipoproteins is shown in Table 3, which includes the available data for other animals of evolutionary relevance. Each lipoprotein from

Centrophorus contains an appreciable amount of hydrocarbon and of alkyldiacylglycerol. The triacylglycerol content quoted for *Scyliorhinus* may also have included some alkylglycerol, since the methods used at that time would not have detected its presence. These glycerol ethers are thought to be absent from the other animals.

As explained in the Experimental section, the chromatographic system used to separate wax esters also resolves the cholesterol esters into two parts. The proportions of these, together with the small amounts of mono- and di-acylglycerols which formed the fraction V eluted from silicic acid columns, are shown in Table 4. All are quoted as a weight percentage of the original lipoprotein.

The proportion of wax ester was too small for precise measurement. In the most favourable analysis, only 13.2% of the fatty acids from fraction CE2 could be attributed to the possible presence of waxes. When expressed as cetyl palmitate, this would be equivalent

Table 3. *Percentage composition (by weight) of VLD, LD and HD lipoproteins from C. squamosus*

Values are the means $\pm$ S.E.M. of four analyses of a pool of shark serum. There was insufficient of the components asterisked to allow replication. Results are also given for the animals listed in Table 1. Abbreviations: PL, phospholipid; UC, unesterified cholesterol; CE, cholesterol ester; TG, triacylglycerol; MAGE, monoalkyldiacylglycerol; HC, hydrocarbon.

	Protein	PL	UC	CE	TG	MAGE	HC
VLD lipoprotein							
<i>Centrophorus</i>	3.1 ± 0.17	15.3 ± 0.10	6.7 ± 0.04	21.4 ± 0.11	23.0 ± 0.23	8.2 ± 0.13	18.8 ± 1.1
<i>Latimeria</i>	14.4	11.5	7.0	3.1	64.0	—	—
<i>Myxine</i>	12.1	17.3	5.1	2.5	48.2	10.6	4.4
<i>Scyliorhinus</i>	28.4	12.4	7.4	8.3	43.5	—	—
<i>Natrix</i>	11.4	14.4	7.9	4.6	61.7	—	—
LD lipoprotein							
<i>Centrophorus</i>	17.6 ± 0.44	20.5 ± 0.33	8.4 ± 0.03	25.9 ± 0.10	8.7 ± 0.09	10.9 ± 0.22	5.9 ± 0.46
<i>Latimeria</i>	27.9	13.6	5.8	3.1	49.7	—	—
<i>Myxine</i>	21.1	22.4	9.6	3.0	29.5	8.7	5.7
<i>Scyliorhinus</i>	29.8	14.8	12.5	22.8	20.1	—	—
<i>Rana</i>	25.4	16.8	13.9	42.5	1.4	—	—
<i>Natrix</i>	28.5	17.7	13.0	35.0	5.8	—	—
HD lipoprotein							
<i>Centrophorus</i>	47.7 ± 0.63	14.5 ± 0.33	3.8 ± 0.01	13.9 ± 0.06	3.4 ± 0.04	8.0*	6.9*
<i>Latimeria</i>	77.1	7.0	2.2	1.3	12.5	—	—
<i>Myxine</i>	42.1	29.6	9.6	0.7	10.5	1.5	6.1
<i>Scyliorhinus</i>	53.1	9.8	9.4	15.6	12.2	—	—
<i>Rana</i>	51.0	13.1	10.3	22.4	3.1	—	—
<i>Natrix</i>	40.0	24.7	7.3	24.4	3.5	—	—

Table 4. *Proportions of short- and medium-chain cholesterol esters (CE1), long-chain polyunsaturated cholesterol esters (CE2), diacylglycerol and monoacylglycerol in Centrophorus lipoproteins*

Results are expressed as the mean percentages (by weight) of the original lipoprotein obtained from four analyses of a pool of serum $\pm$ S.E.M.

	CE1	CE2	Diacylglycerol	Monoacylglycerol
VLD lipoprotein	9.6 ± 0.04	11.8 ± 0.07	2.0 ± 0.07	1.6 ± 0.04
LD lipoprotein	12.6 ± 0.07	13.3 ± 0.03	1.3 ± 0.03	0.9 ± 0.04
HD lipoprotein	5.9 ± 0.03	8.0 ± 0.03	1.2 ± 0.04	0.6 ± 0.02

Table 5. *Fatty acid composition of the lipid esters of serum lipoproteins from C. squamosus*

The procedure used for analysis of the fatty acids by g.l.c. is described under 'Methods'. Results are expressed as a weight percentage of recovered fatty acids; the starting material was a single pool of serum. The technical error (T.E.) of determination of each fatty acid (except those marked N.D.) was measured separately, and calculated from the equation $T.E. = \sqrt{\frac{\sum d^2}{2N}}$ where d is the difference between duplicate determinations, and N is the number of duplicates. Abbreviations are as in Table 3.

Fatty acid	T.E.	VLD lipoprotein					LD lipoprotein					HD lipoprotein				
		CE1	CE2	TG	MAGE	PL	CE1	CE2	TG	MAGE	PL	CE1	CE2	TG	MAGE	PL
C _{14:0}	±0.17	2.8	0.2	2.0	1.7	1.0	2.2	0.3	1.5	1.6	1.9	2.0	0.3	2.7	1.8	1.3
C _{16:0}	±0.19	11.4	2.0	18.9	20.7	42.6	12.1	1.9	17.8	16.2	35.6	14.6	2.9	23.0	18.6	32.1
C _{16:1}	±0.20	8.8	0.7	6.8	4.9	3.7	8.0	0.6	6.0	3.7	3.6	7.5	1.0	7.7	4.4	3.4
C _{18:0}	±0.15	2.8	0.9	3.5	3.4	2.8	3.3	1.1	3.8	3.3	3.6	3.9	1.6	4.7	5.9	4.9
C _{18:1}	±0.15	62.9	1.9	39.5	36.2	23.4	58.2	1.1	32.8	26.7	19.0	55.3	1.4	35.4	27.0	21.0
C _{18:2}	±0.29	2.6	0.6	0.8	3.6	1.7	3.6	0.6	1.2	2.0	1.9	3.5	0.6	1.6	10.1	1.4
C _{18:3}	±0.30	—	0.6	—	—	—	—	0.7	—	—	—	—	0.6	—	1.2	—
C _{20:1}	±0.15	5.6	—	5.8	7.5	0.8	6.3	—	5.7	8.7	1.4	5.7	—	4.6	6.6	1.3
C _{20:4}	±0.40	1.4	9.7	—	—	4.7	3.7	11.9	1.7	—	4.5	—	11.8	—	—	5.4
C _{20:5}	±0.34	—	37.5	4.3	—	7.1	—	38.1	4.8	2.5	7.5	—	36.2	4.0	1.9	7.2
C _{22:1}	±0.70	—	—	4.5	8.2	—	—	—	4.5	11.9	—	—	5.2	3.3	7.6	—
C _{22:4}	N.D.	—	—	—	—	—	—	1.2	—	—	—	—	1.8	—	—	3.0
C _{22:5}	N.D.	—	8.6	—	—	—	—	5.4	—	2.3	—	—	4.8	—	—	—
C _{22:6}	±0.57	—	33.4	12.4	11.4	11.0	—	32.8	17.3	14.2	18.5	—	32.8	10.3	11.8	16.6
Unidentified	—	1.8	4.0	1.5	2.5	1.2	2.6	4.3	3.1	7.0	2.6	2.4	4.0	2.8	3.1	2.3

Table 6. *Percentage distribution by weight of monoalkyl ethers of glycerol derived from the serum lipoproteins of C. squamosus*
Analyses were made on a single pool of serum by the procedures described under 'Methods'. N.I., unidentified substances of equivalent alkyl-chain length less than 16 carbon atoms.

Alkyl group ...	Percentage distribution						N.I.
	C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{20:1}	
VLD lipoprotein	0.6	13.1	3.8	19.4	52.8	4.7	5.6
LD lipoprotein	0.5	18.5	3.4	16.4	41.5	10.9	8.9
HD lipoprotein	0.7	23.4	—	13.8	34.5	13.6	14.1

to approx. 1.2% of the original lipoprotein by weight. However, as other experiments gave even lower values, this can probably be regarded as an upper limit.

Since squalene is a well-substantiated constituent of the oil from the livers of a variety of sharks, and has also been found in their serum (Sargent *et al.*, 1973), it seemed likely that it would form at least a part of the hydrocarbon fraction of the *Centrophorus* serum lipoproteins. This hypothesis was supported by the observation that the hydrocarbon gave a single peak, inseparable from that of authentic squalene, by g.l.c. We were unable to detect pristane, a hydrocarbon which is often present in marine organisms (Malins & Wekell, 1971).

Distribution of fatty acids and monoalkylglycerols

The distribution of the fatty acids in the four major classes of lipid esters is given in Table 5. The fractions CE1 and CE2 are given separately and clearly show the predominance of the longer-chain acids in the latter. Oleic acid was prominent in each of the other fractions. There was also a substantial amount of palmitic acid, particularly in the phospholipids. The C_{22:6} component was likewise significant in all fractions except CE1. There was insufficient of the partial glycerides for the distribution of their fatty acids to be satisfactorily determined.

The distribution of the glycerol monoethers obtained by hydrolysis of the native diacyl derivatives was characterized by the predominance of the chimyl (C_{16:0}), batyl (C_{18:0}) and selachyl (C_{18:1}) alcohols (Table 6). These three comprised more than 70% of the whole ether fraction. The proportion of selachyl alcohol decreased considerably from the VLD through the LD to the HD lipoproteins, whereas the proportion of chimyl alcohol increased. A significant quantity of an alcohol, identified by carbon-number estimations (Ackman, 1969) as the C_{20:1} compound, was also present. This amounted to 4.7% of the alcohols isolated from the VLD lipoproteins, increasing to 13.6% of those from the HD lipoproteins. Several other compounds of lower molecular weight were also present, which we could not identify.

Fig. 1. *Electrophoretic mobility on polyacrylamide gel of the Centrophorus serum apolipoproteins which are soluble in 8M-urea*

Electrophoretic mobility is expressed as the ratio of the distance of migration of each apoprotein to that of the dye front. Electrophoresis was carried out in 7.5% (w/v) polyacrylamide gel containing 8M-urea. The values quoted are the means for duplicate gels run on three different lipoprotein preparations.

Properties of the apolipoproteins

The amino acid compositions of apo-VLD lipoprotein, apo-LD lipoprotein and apo-HD lipoprotein are presented in Table 7. In each case the dicarboxylic acids were predominant, accounting for about 25% of the total protein. Leucine, serine and threonine were each present to the extent of about 8–10%, whereas methionine and histidine both amounted to only about 1%.

The apoproteins from each of the three classes of lipoproteins were completely soluble only in solutions of sodium decyl sulphate (1.0%, w/v). When the

Table 7. *Amino acid composition of Centrophorus serum lipoproteins*

Duplicate analyses were made of two separate protein preparations in each case, except for the fraction I, of which there was sufficient for only two analyses. Values are expressed as mol/100 mol of amino acid recovered (means $\pm$ S.E.M). The values for human apolipoprotein B are taken from Gotto *et al.* (1972).

Amino acid	Apo-VLD lipoprotein	Apo-LD lipoprotein	Apo-HD lipoprotein	Apo-LD lipoprotein fraction I	Human apo-lipoprotein B
Lysine	5.5 $\pm$ 0.40	6.8 $\pm$ 0.25	6.1 $\pm$ 0.47	9.3	8.0
Histidine	1.1 $\pm$ 0.33	1.1 $\pm$ 0.45	1.6 $\pm$ 0.58	2.4	2.2
Arginine	3.7 $\pm$ 0.13	3.2 $\pm$ 0.06	3.8 $\pm$ 0.17	4.2	3.1
Aspartic acid	13.0 $\pm$ 0.24	12.8 $\pm$ 0.40	11.0 $\pm$ 0.51	11.5	10.9
Threonine	7.8 $\pm$ 0.04	8.0 $\pm$ 0.27	7.6 $\pm$ 0.20	7.3	6.7
Serine	9.3 $\pm$ 0.26	8.7 $\pm$ 0.29	10.6 $\pm$ 0.22	9.5	8.7
Glutamic acid	12.9 $\pm$ 0.14	11.9 $\pm$ 0.16	14.5 $\pm$ 0.55	11.5	10.8
Proline	4.4 $\pm$ 0.10	4.4 $\pm$ 0.18	4.7 $\pm$ 0.42	3.0	4.2
Glycine	5.9 $\pm$ 0.08	4.9 $\pm$ 0.04	7.8 $\pm$ 0.75	7.0	5.5
Alanine	6.7 $\pm$ 0.30	6.1 $\pm$ 0.05	6.9 $\pm$ 0.08	6.6	7.0
Valine	5.6 $\pm$ 0.14	5.6 $\pm$ 0.20	4.2 $\pm$ 0.71	5.3	5.6
Methionine	1.2 $\pm$ 0.09	1.2 $\pm$ 0.10	1.0 $\pm$ 0.02	0.9	1.8
Isoleucine	5.5 $\pm$ 0.19	6.2 $\pm$ 0.09	4.5 $\pm$ 0.02	5.3	5.0
Leucine	10.2 $\pm$ 0.27	11.0 $\pm$ 0.26	9.4 $\pm$ 0.14	9.7	12.3
Tyrosine	3.4 $\pm$ 0.15	3.5 $\pm$ 0.10	3.6 $\pm$ 0.18	2.8	3.3
Phenylalanine	3.9 $\pm$ 0.10	4.3 $\pm$ 0.02	2.9 $\pm$ 0.13	3.8	4.9

solution of apo-LD lipoprotein was chromatographed on Sephadex G-200 in the presence of the detergent, about 90% of the protein (referred to as fraction I) was eluted at the void volume. The amino acid composition of this fraction is also shown in Table 7, and is generally like that of the apo-LD lipoprotein. However, fraction I was richer in lysine, but we cannot assess the significance of this observation.

The degree of heterogeneity and the molecular weight of apo-LD lipoprotein was estimated by electrophoresis in sodium dodecyl sulphate/polyacrylamide gel. This resolved the apoprotein into about 11 components (Plate 1a). The most prominent was a group of four closely spaced bands with a lower limit of 250000 to their molecular weight. Below these ran a more widely spaced series of about seven faint bands, extending down to mol.wt. 150000.

Exhaustive extraction of the apo-VLD, apo-LD and apo-HD lipoproteins with 8M-urea in Tris/HCl buffer (pH 8.9) yielded a solution of polypeptides which, unlike fraction I, could penetrate a 7.5% polyacrylamide gel. By electrophoresis in this gel/buffer combination, the mixture could be resolved into ten components (Plate 1b, Fig. 1). The patterns obtained from all three apolipoprotein fractions had a prominent group of four components, with electrophoretic mobilities between 0.52 and 0.70, which formed bands 7–10. Of these, band 8 (relative mobility 0.58) was the strongest in each case. The sixth band, with a relative mobility of 0.44, was always very faint. Bands 4 and 5 were of moderate intensity, but tended to be diffuse and often poorly resolved. In the apo-VLD lipoprotein pattern, the remaining slow com-

ponents were all faint, but in the apo-LD lipoproteins band 3 was prominent, although bands 1 and 2 were weak. By contrast, in the apo-HD lipoproteins, it was band 1 that was intense, whereas bands 2 and 3, although distinct, were markedly weaker.

Discussion

Centrophorus squamosus, like other vertebrates, has serum lipoproteins which extend over the whole range of densities from 1.2 to 0.94 g/ml. In their morphology and range of size, as disclosed by electron microscopy, these particles are indistinguishable from those of mammals (Forte *et al.*, 1968; Puppione *et al.*, 1970, 1971; Sardet *et al.*, 1972; Scanu *et al.*, 1973). In particular, the fraction of density 1.006–1.063 g/ml has a particle-size distribution very similar to that of the corresponding human preparation (Forte *et al.*, 1968). The ultracentrifugally determined distribution differs, however, from that usually found in mammals in that the VLD lipoproteins (S_r 20–400) are the main class, accounting for some 61% of the total. Of the remainder, approx. 34% are in the LD lipoprotein class (S_r 0–20) and 5–6% in the HD lipoprotein fraction. This type of pattern is not uncommon among fish and reptiles, however (Mills & Taylaur, 1971, 1973), and in this respect *C. squamosus* is like the coelacanth (*Latimeria chalumnae*), the hagfish (*Myxine glutinosa*) and the conger eel (*Conger vulgaris*), but differs from the dogfish (*Scyliorhinus canicula*) and plaice (*Pleuronectes platessa*), in which the concentration of VLD-lipoprotein compounds is low. The content of

EXPLANATION OF PLATE I

(a) Patterns of human and *Centrophorus* apo-LD lipoproteins in sodium dodecyl sulphate/polyacrylamide gel and

(b) patterns of *Centrophorus* apolipoproteins in 8M-urea/polyacrylamide gel

(a) Electrophoresis was performed in 3.3% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate for about 4 h at 8 mA/gel. The molecular weights of the stained protein bands were calculated as described by Weber & Osborn (1969). Purified proteins of known molecular weight were run in each set of gels for calibration purposes. (A) Human apo-LD lipoprotein derived from a fraction $d = 1.024-1.045$. (B) *Centrophorus* apo-LD lipoprotein derived from the fraction $d = 1.007-1.063$. (b) Extracts of apolipoprotein preparations in 8M-urea at pH 8.0 were run in 7.5% (w/v) polyacrylamide gel containing 8M-urea at pH 8.0. About 100 μ g of protein was applied in each case: gels were stained with Coomassie Blue. (A) VLD lipoprotein, $d = 1.007$; (B) LD lipoprotein, $d = 1.007-1.063$; (C) HD lipoprotein, $d = 1.063-1.21$.

chylomicrons in the serum of *Centrophorus* was quite low, the VLD lipoproteins extending only to the region of S_r 700.

The lipoproteins of *Centrophorus* contain all the components that are normally found in the lipoproteins of vertebrates, but are distinguished by having, in addition, significant proportions of hydrocarbon and alkyl diacylglycerols. Although evidence is at present meagre, it appears that these two compounds are rare in higher vertebrates, including reptiles and teleosts. However, the work of Sargent *et al.* (1973) implies that they are present in the lipoproteins of some other species of shark. The reason for the large amounts of hydrocarbons and glycerol ethers in sharks is not fully understood, but is thought to be concerned with the control of buoyancy (Corner *et al.*, 1969). Since elasmobranchs have no swim bladder, about 30% of their weight must be lipid if they are to be weightless in water (Love, 1970). Alkyl diacylglycerols can be metabolized to yield energy, but at a much lower rate than are triacylglycerols (Sargent *et al.*, 1973). This may reflect the need to conserve the ethers as buoyant agents (Malins & Barone, 1970), but does not exclude their possible role as a reserve lipid. Although hydrocarbons contribute to the buoyancy reserve in sharks, it is significant that they are also present in certain insects (Thomas & Gilbert, 1968), as well as in the phylogenetically more primitive hagfish (Table 3) and sturgeon (C. E. Tylaur, unpublished work). It is possible therefore that they are a feature of 'primitive' lipid metabolism which has been lost during the evolution of teleosts and higher classes of animals.

The incorporation of the unusual ether and hydrocarbon components into the lipoprotein structure seems to be achieved by an adjustment which is different in each of the three main lipoprotein classes. The VLD lipoproteins from *Centrophorus* are remarkable for their unusually low proportion of triacylglycerol and their high content of cholesterol. This is a combination which could be accounted for if the mean density of the lipoproteins was close to 1.006 g/ml (i.e. near the S_r 20 boundary). In fact, however, some 38% of the VLD lipoproteins were in the S_r 100–400 fraction, which approaches the proportion found in man, namely 50% (Mills & Tylaur, 1971). Moreover, if the distribution was heavily weighted towards the particles of density 1.006 g/ml, the protein content would also be high (Gustafson *et al.*, 1965), whereas in *Centrophorus* it is close to that usually associated with chylomicrons. Compared with the corresponding compounds from higher animals, the shark lipoproteins therefore seem genuinely to be deficient in triacylglycerol. However, the total of triacylglycerol, glycerol ethers and hydrocarbons amounts to 53.5% of the *Centrophorus* VLD lipoproteins, which is close to the proportion of triacylglycerol in the VLD lipoprotein compounds of

higher animals. This seems to suggest that some of the usual complement of triacylglycerol has been replaced in the shark lipoproteins by the ethers and hydrocarbons.

Nevertheless, it may also be observed that VLD lipoproteins, which are unusually rich in cholesterol but deficient in triacylglycerol and protein, are found in patients with Type-III hyperlipoproteinaemia (Hazzard *et al.*, 1970), and in guinea pigs and rats which have been fed on a diet enriched with cholesterol (Mills *et al.*, 1972; Lasser *et al.*, 1973). Furthermore, the ratio of esterified to unesterified cholesterol is raised in these abnormal human lipoproteins, as it is in the ones from *Centrophorus*. Although it is at present impossible to explain this peculiarity of the shark lipoproteins, the presence of large amounts of squalene in the tissues (Heller *et al.*, 1957) suggests that this fish may have a considerable potential for synthesizing cholesterol, which could lead to a metabolic situation akin to that in the cholesterol-fed animals.

By contrast with the VLD lipoproteins, the incorporation of alkyl diacylglycerols and hydrocarbons into the LD particles appears to be at the expense of both cholesterol ester and triacylglycerol, since these two lipids both contribute less than the average found in other animals, and together account for only 34.1% of the shark LD lipoprotein instead of the 48.8, 51.9 or 43.6% which is the average for mammals, birds and reptiles/fish respectively (Mills & Tylaur, 1971).

In the HD lipoproteins, there is no marked diminution of any one of the usual four lipid components, though all are a few per cent below the average for fish and reptiles (Mills & Tylaur, 1971). It may be significant therefore that their total contribution of 35.6% is less than the average by 17.0%, which is close to the 14.9% which is the total of the alkyl diacylglycerols and hydrocarbons taken together. Thus the incorporation of glycerol ethers and hydrocarbons into the structure of the HD lipoproteins seems to be possible only if a fraction of the entire lipid moiety is displaced, whereas in the larger VLD and LD lipoproteins they may be accommodated within the lipid core.

It is of some interest to know whether the presence of unusual lipids in the *Centrophorus* lipoproteins requires the presence of unusual protein components to stabilize the structure. Unfortunately, apart from a brief report presented by Skinner (1973) on the proteins of cod serum HD lipoproteins, and a study of the apo-HD lipoproteins of the pink salmon (*Oncorhynchus gorbuscha*) by Nelson & Shore (1974), little work has hitherto been done on the apolipoproteins of fish. In both these cases, the pattern of apoproteins was like that found in the HD lipoproteins of man (Scanu *et al.*, 1969), rat (Koga *et al.*, 1971) and guinea pig (Chapman *et al.*, 1975). In

Centrophorus, too, there are soluble apoproteins with electrophoretic mobilities like those of the apoproteins from higher animals. Thus the prominent group of fast-moving peptides has a range of mobilities like those of the human CII and CIII peptides; band 5 occupies a position near that of the band V of guinea-pig apoproteins (Chapman *et al.*, 1975), and band 3 has a mobility like that of a human A apoprotein.

Quantitatively, the proportions of the soluble apoproteins in the VLD and LD lipoproteins from *Centrophorus* are not unlike those from the corresponding lipoproteins of other animals. On the other hand, the pattern of apo-HD lipoproteins is unusual in several respects. For example, the fast-moving bands 7–10 are more prominent than is usual in HD lipoproteins. Moreover, there is only a small contribution from peptides in the region where the A apoproteins are an obvious feature of the pattern in other animals. Instead, there is an intense component (band 1) with a mobility like that of the Band X of guinea pig (Chapman *et al.*, 1975). By electrophoretic analysis, therefore, the pattern of proteins in the HD lipoproteins differs from that found in other animals to a greater extent than do the patterns of the VLD and LD apolipoproteins. However, lack of material, and in particular the low content of the HD lipoprotein in *Centrophorus*, has prevented us from making amino acid analyses of any of these soluble apoproteins, and from testing their ability to activate lipoprotein lipase or lecithin-cholesterol acyltransferase. We therefore cannot comment on their chemical analogy to the apoproteins of man or other animals.

However, the major apolipoprotein of the shark is the high-molecular-weight fraction I. This is soluble only in detergents such as sodium dodecyl sulphate and is akin to the human apolipoprotein B in both its amino acid composition and its physical properties. *Centrophorus* is therefore like a number of mammals and birds in which counterparts to apolipoprotein B have been observed (Bersot *et al.*, 1970; Koga *et al.*, 1971; Chapman *et al.*, 1975; Mahley *et al.*, 1976; Kelley & Alaupovic, 1976). This suggests that the organization of the fish lipoproteins is broadly similar to that of lipoproteins from higher animals, despite the presence of hydrocarbons and glycerol ethers. Nonetheless, the shark apolipoprotein does not react with antisera against human apolipoprotein B (S. Goldstein, private communication), and it also differs from the human protein in its content of leucine. Whether these differences are adjustments necessary to accommodate the unusual lipids cannot yet be determined.

It is noteworthy that we have been unable to detect more than trace amounts of waxes in the serum lipoproteins of *Centrophorus squamosus*. Although the presence of these compounds in marine animals is

apparently well established by numerous observations (cf. Malins & Wekell, 1971), there remains a good deal of uncertainty about their validity in particular cases. For example, Malins & Wekell (1971) reported the presence of substantial amounts of wax esters in the livers of deep-sea sharks, including species of *Centrophorus*, *Centroscymnus* and *Deania*. By contrast, Sargent *et al.* (1973) found no wax ester in the liver or muscle of *Centroscymnus*, *Deania* or *Squalus acanthias*. However, they reported the presence of waxes in the sera of all three of these sharks. It seems likely that some of this confusion may have arisen through the separation of cholesterol esters into the two fractions we have called CE1 and CE2, since the latter runs in the same position as wax esters on t.l.c. It may be relevant in this context that Sargent *et al.* (1973) remark on the unusually high proportion of oleic acid that they found in the cholesterol esters of *S. acanthias*, since a high proportion of this acid is typical of fraction CE1.

Although few detailed studies of fish lipoproteins have been made, it is clear that their chemistry varies more than that of mammalian lipoproteins. Both the primitive hagfish and the coelacanth have lipoproteins in which the proportion of cholesterol is low, with more unesterified cholesterol than esterified (Mills & Taylaur, 1973). In the pink salmon (*O. gorbuscha*), on the other hand, the HD lipoproteins (which were the only ones available for examination) were remarkably like those of man in chemical composition, with a ratio of esterified to unesterified cholesterol of almost 6:1 (Nelson & Shore, 1974). Between these two extremes lie *Sardinops caerulea* (Lee & Puppione, 1972), *Scyliorhinus canicula* (Mills & Taylaur, 1971) and *Centrophorus squamosus*, in which the ratio ranges from 1:1 to 3:1. In *Centrophorus* we have suggested that this is partly due to replacement by other lipids, but it is not certain whether this is also true of the other two fish. However, in trout lipoproteins we have found the same ratio of esterified to unesterified cholesterol, although waxes, hydrocarbons and alkyldiacylglycerols were almost entirely absent (C. E. Taylaur, unpublished work). This diversity of composition seems to suggest that the mechanism of lipoprotein biosynthesis in fish is rather variable. Nonetheless, the particles formed do not seem to differ materially in shape, size or general form from those produced in mammals by what may be a more well-defined pathway. Detailed studies of fish lipoproteins may therefore be a promising way of increasing our understanding of the structure and synthesis of lipoproteins.

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