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Plasma lipoprotein and apolipoprotein distribution as a function of density in the rainbow trout (*Salmo gairdneri*)

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I have previously described [Babin (1987) J. Biol. Chem. **262**, 4290–4296] the apolipoprotein composition of the major classes of trout plasma lipoproteins. The present work describes the use of an isopycnic density gradient centrifugation procedure and sequential flotation ultracentrifugation to show: (1) the presence of intermediate density lipoproteins (IDL) in the plasma, between 1.015 and 1.040 g/ml; (2) the existence of a single type of M_r 240000 apoB-like in the low density lipoproteins (LDL, $1.040 < \rho < 1.085$ g/ml); (3) the presence of apoA-I-like (M_r 25000) in the densest LDL; (4) the adequacy of 1.085 g/ml as a cutoff between the LDL and high density lipoproteins (HDL); (5) the accumulation of M_r 55000 and 76000 apolipoproteins and apoA-like apolipoproteins in the 1.21 g/ml infranatant. The fractionation of trout lipoprotein spectrum thus furnishes the distribution of the different lipoprotein classes and leads to the description of the constituent apolipoproteins, which account for about 36% of circulating plasma proteins in this species.

INTRODUCTION

The different classes of plasma lipoproteins in fish have been classified analogously to those of mammals (see [1] for review). The plasma concentration, as well as the chemical composition, in particular lipids, of the individual lipoprotein classes isolated by sequential flotation ultracentrifugation have been described previously [2–7]. Following the initial characterizations of apolipoproteins in salmonids [2,3,8] we recently furnished a detailed description of the apolipoprotein composition of the major classes of trout lipoproteins [9].

The aim of the present work was to define the distribution of the different classes of trout lipoproteins as a function of their density. In addition to isolating them by sequential flotation ultracentrifugation, they were fractionated by the use of an isopycnic density gradient ultracentrifugation procedure. These procedures lead to an overall and continuous view of the lipoprotein and apolipoprotein profile as a function of the physicochemical properties of the particles.

MATERIALS AND METHODS

Plasma

Female trout in previtellogenesis were raised at constant temperature (10–12 °C) and nourished *ad libitum* with granules (Aqualim) containing 8% lipids. They were anaesthetized with ethyl carbamate (3 g/l) and blood was removed over 0.15% EDTA + 0.015% NaN_3 by cardiac puncture with a fine catheter. Plasma was obtained by centrifugation (3000 *g*, 10 min).

Isolation of lipoproteins

Lipoproteins were obtained by sequential ultracentrifugal flotation [10] at 10 °C using a Beckman L8-70 ultracentrifuge and a 70-Ti rotor as previously described [9,11]. A density gradient ultracentrifugation procedure [12,13] was used for the fractionation of whole plasma lipoproteins. Discontinuous six-step gradients were obtained with NaBr solutions, with successive densities (from top to bottom of tube) of 1.006 g/ml (0.8 ml), 1.019 g/ml (2.5 ml), 1.063 g/ml (3.5 ml), 1.21 g/ml (2.5 ml), 1.31 g/ml (1 ml of plasma adjusted to this density with solid NaBr) and 1.386 g/ml (0.5 ml). Control gradients were also constructed using 1 ml of NaBr solution of $\rho = 1.31$ g/ml instead of the adjusted plasma sample. The nonprotein solvent density of trout plasma is 1.015 g/ml [3]. The gradients were then placed in a Beckman SW 41-Ti swinging bucket rotor ($r_{av.}$ 110.2 mm) and centrifuged at 180000 *g* for 24 h at 10 °C in a Beckman L8-70 ultracentrifuge; no braking was used at the end of the run. The gradients were then divided into successive 0.6 ml fractions from the meniscus downwards by aspiration with a micropipette. The background density of each fraction was determined at 20 °C by reference to the density profile obtained from control gradients. The fractions were then exhaustively dialysed against 0.15 M-NaCl/1 mM-EDTA/30 mM-NaN_3 at 4 °C (pH 7.4). The protein concentration was determined with a modified Lowry assay [14] and the preparations were examined with a JEOL 100 B electron microscope (80 kV) after they were negatively stained with 2% phosphotungstic acid (pH 7.3).

Electrophoretic methods

Polyacrylamide-gel electrophoresis of plasma lipoproteins was performed in discontinuous three-step gradients [15] after prestaining the lipoproteins with Sudan Black B. Gels were made with loading gel at 2% (pH 6.7), stacking gel at 3.25% (pH 6.7) and running gel at 3% (pH 8.9). After electrophoresis the gels were scanned with a laser densitometer (LKB 2202). Continuous gradient slab gel electrophoresis of plasma lipoproteins was performed in linear gradients of 2–12% polyacrylamide adjusted to pH 8.3 with Tris/glycine

Abbreviations used: apo, apolipoprotein; VHDL, HDL, LDL, IDL and VLDL, very-high-density, high-density, low-density, intermediate-density and very-low-density lipoproteins.

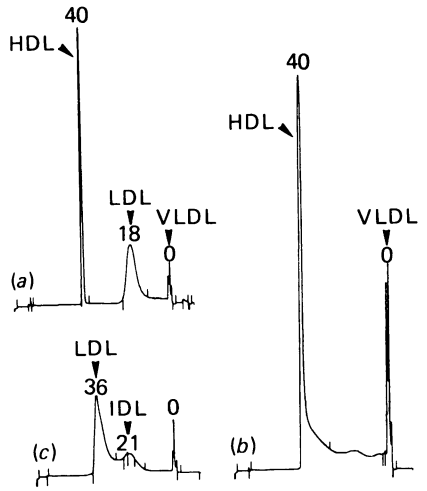


Fig. 1. Densitometric scans of trout and human plasma lipoproteins separated by electrophoresis on discontinuous polyacrylamide gels

(*a*) Total human plasma lipoproteins ($\rho < 1.21$ g/ml). (*b*) Total trout plasma lipoproteins ($\rho < 1.21$ g/ml). (*c*) Trout IDL+LDL ($1.015 < \rho < 1.085$ g/ml). Samples were prestained with Sudan Black B for the detection of lipids and the identity of lipoproteins was determined by electrophoresis of purified classes obtained by sequential ultracentrifugation flotation. The origin of the migration distance is the separation between the loading gel and the stacking gel.

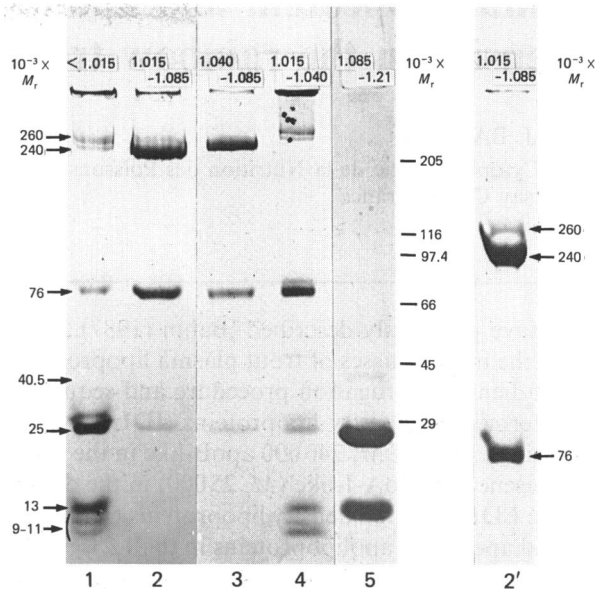


Fig. 2. Electrophoretic patterns in SDS/glycerol/polyacrylamide gel of the apolipoproteins of the principal classes of trout plasma lipoproteins isolated by sequential ultracentrifugation flotation from a trout 24 h after feeding

Electrophoresis in linear gradient of 3.5–15% polyacrylamide and 8–12% glycerol. Gel was stained with Coomassie Blue R250. Track 1, apo-VLDL ($\rho < 1.015$ g/ml); track 2, apo-IDL+apo-LDL ($1.015 < \rho < 1.085$ g/ml); track 3, apo-LDL ($1.040 < \rho < 1.085$ g/ml); track 4, apo-IDL ($1.015 < \rho < 1.040$ g/ml); track 5, apo-HDL ($1.085 < \rho < 1.21$ g/ml). The M_r values ($\times 10^{-3}$) of the apolipoproteins are on the left and of the standards on the right. Track 2', apo-IDL+apo-LDL ($1.015 < \rho < 1.085$ g/ml) after electrophoresis in a linear gradient of 3.5–8% polyacrylamide and 8–12% glycerol.

buffer (2 M); 30 μg of protein was applied to each well and electrophoresis was carried out at 160 V for 18 h in a Tris/borate buffer [0.09 M-Tris/0.08 M-boric acid/3 mM-EDTA/3 mM-NaN_3 (pH 8.35). Gels were subsequently stained with 0.5% Sudan Black B in 60% ethanol for 1 h and diffusion-destained in 5% acetic acid. Apolipoproteins were electrophoresed under reducing conditions on SDS/glycerol/polyacrylamide slab gels in a linear gradient of 3.5–15% polyacrylamide and 8–12% glycerol [9]. The M_r values of apolipoproteins were determined as previously described [9] by comparison with a simultaneous electrophoresis of proteins of known M_r. The reference curve was constructed from the densitometer scan of the gel stained with Coomassie Blue R 250. The standards used here were myosin (M_r 205000), β-galactosidase (M_r 116000), phosphorylase *b* (M_r 97000), albumin (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000), trypsin inhibitor (M_r 20100) and lactalbumin (M_r 14400).

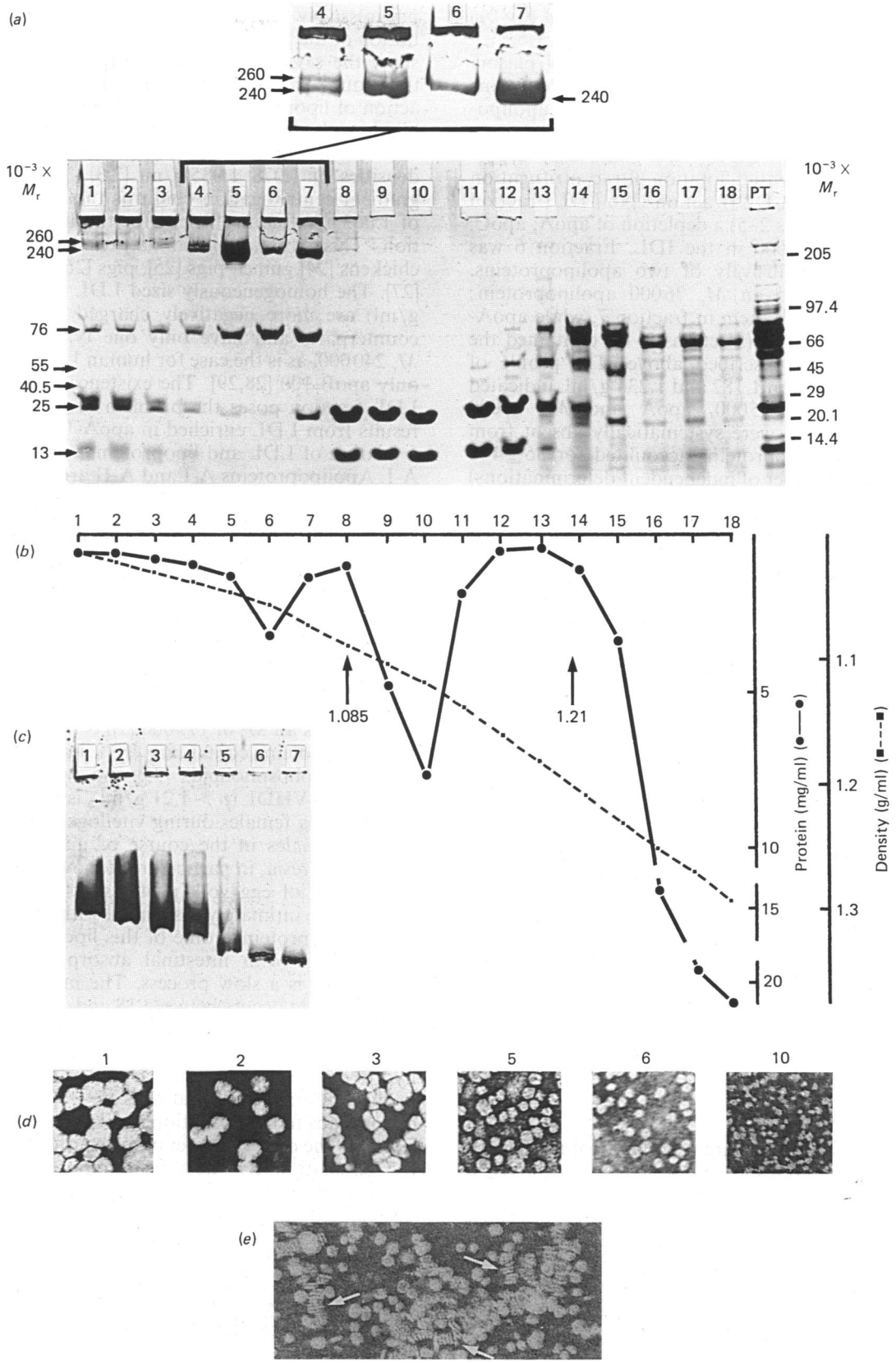
RESULTS

The total lipoprotein profile of adult trout shows the predominance of HDL (Fig. 1*b*) (48 h after feeding). This is not the case in man, where LDL are the majority species (Fig. 1*a*). The fraction isolated at $1.015 < \rho < 1.085$ g/ml (Fig. 1*c*) in trout could be resolved into two major classes: IDL and LDL. Trout LDL have a much greater anodic migration than their human counterparts.

Fig. 2 shows the apolipoprotein composition of the different lipoprotein classes isolated by sequential flotation ultracentrifugation. The VLDL (containing chylomicrons) ($\rho < 1.015$ g/ml, track 1) and the IDL ($1.015 < \rho < 1.040$ g/ml, track 4) contained two high-M_r apolipoproteins (apoB-like) of M_r 260000 and 240000, which was also the case for the IDL+LDL fraction ($1.015 < \rho < 1.085$ g/ml, tracks 2 and 2'). In the LDL ($1.040 < \rho < 1.085$ g/ml, track 3), however, only the species of M_r 240000 was present. The M_r 76000 apolipoprotein was proportionally greater in LDL than in VLDL and IDL, as a result of the presence of M_r 25000 (apoA-I-like), M_r 13000 (apoA-II-like) and M_r 9000–11000 (apoC-like) apolipoproteins in these lipoproteins. The HDL ($1.085 < \rho < 1.21$ g/ml, track 5) contained four apolipoproteins, two major species with M_r 25000 [apoA-I-like (65%)] and M_r 13000 [apoA-II-like (33%)] and two

Fig. 3. Density gradient study of the lipoprotein and apolipoprotein distribution in the plasma of the rainbow trout 28 h after feeding

(*a*) Electrophoretic patterns in SDS/glycerol gel slabs (linear gradient of 3.5–15% polyacrylamide and 8–12% glycerol) of trout apolipoproteins and plasma proteins from gradient subfractions 1–18. The gradient was divided into 18 successive 0.6 ml fractions from the meniscus downwards by aspiration with a micropipette. The M_r values ($\times 10^{-3}$) of the apolipoproteins are on the left and of the standards on the right. PT, total plasma of the same trout. Staining with Coomassie Blue R250. (*b*) ■---■, Density profile determined from control density gradient subfractions containing only NaBr solutions (means of three gradients)



after ultracentrifugation for 24 h; ●—●, lipoprotein profile as evaluated by protein content in each fraction of the density gradient. The abscissa is the number of successive fractions from the top of the tube. (*c*) Electrophoresis of native lipoprotein gradient subfractions (1–7) on polyacrylamide gel slabs containing a linear gradient of 2–12% polyacrylamide, pH 8.3. Staining with Sudan Black B. (*d*) Electron micrographs of negatively stained trout plasma lipoproteins isolated by density gradient centrifugation (subfractions 1, 2, 3, 5, 6 and 10). Final magnification × 100 000. (*e*) Electron micrographs of negatively stained trout plasma lipoprotein fraction isolated by sequential ultracentrifugation flotation ($1.026 < \rho < 1.085$ g/ml) 24 h after feeding, showing many disk-shaped lipoproteins assembled in cylinders. Final magnification × 100 000.

minor species (see also Fig. 3*a*) with M_r 55000 (0.8%) and M_r 40500 (1.2%).

Density gradient ultracentrifugation of total plasma was used to investigate the distribution of lipoproteins and their constitutive apolipoproteins. The apolipoprotein profiles of fractions 1, 6 and 10 (Fig. 3*a*) were identical with those of VLDL, LDL and HDL, respectively, isolated by sequential flotation ultracentrifugation (Fig. 2). Going from VLDL (fraction 1) to LDL (fraction 6), we note (in fractions 2–5) a depletion of apoA, apoC, and apoB of M_r 260000 in the IDL. Fraction 6 was composed almost exclusively of two apolipoproteins, M_r 240000 apoB and an M_r 76000 apolipoprotein; apoA-I was found with them in fraction 7, while apoA-II was absent. The HDL (fractions 8–12) contained the four apolipoproteins described above. The profile of fractions 13–15, between 1.182 and 1.234 g/ml, indicated the presence of M_r 55000, apoA and M_r 76000 apolipoproteins which were systematically absent from the HDL. The apolipoproteins accounted for $36 \pm 4\%$ ($n = 10$) (n is the number of independent determinations) of total plasma proteins. The concentration of apoA-I in the plasma was 11.8 ± 3.3 mg/ml ($n = 12$) ($25 \pm 2\%$ of plasma proteins). These values were determined by integrating the peak surfaces of the densitometer scans.

The density gradient profile (Fig. 3*b*) shows that trout LDL are denser than human LDL, included between 1.040 and 1.085 g/ml (fraction 6: $\rho = 1.056$ g/ml). The cutoff density of 1.085 g/ml in sequential flotation ultracentrifugation thus leads to a very good separation of LDL and HDL; the latter could be isolated between 1.085 and 1.21 g/ml, with a peak at $\rho = 1.118$ g/ml (fraction 10).

Lipoprotein separation (fractions 1–7) on polyacrylamide gels in nondenaturing conditions (Fig. 3*c*) shows a considerable heterogeneity between 1.015 and 1.085 g/ml and the existence of IDL of progressively decreasing size, with the LDL distributed in a homogeneous band (fractions 6 and 7). Electron microscopic observation of the particles (Fig. 3*d*) confirmed the heterogeneous distribution of the VLDL (mean 30 nm) and the IDL. The LDL (fraction 6) and HDL (fraction 10) were more homogeneously distributed (respective means of 18 and 8 nm). Disk-shaped lipoproteins assembled in cylinders were occasionally observed in the LDL fraction (Fig. 3*e*).

DISCUSSION

A number of fish species are useful animal models for studying lipid metabolism and transport. In contrast with mammals, which mobilize carbohydrates, fish preferentially utilize lipids as their main source of energy [16]. The plasma profile of salmonid plasma lipoproteins is dominated by HDL, whose concentrations may reach 1500 to 2000 mg/100 ml [3]. This results in a high plasma concentration of apolipoproteins, which account for 36% of total plasma protein in the trout. In this species plasma apoA-I concentration is about 12 mg/ml; the corresponding value in man is 1.3 mg/ml [17].

Plasma VLDL of adult trout have a dual intestinal and hepatic origin, as shown by numerous ultrastructural [18–21] and biochemical [22] studies. The presence of chylomicrons in the plasma is now established [2,3,7,11]. The apolipoprotein composition, similar to that of the VLDL ($\rho < 1.015$ g/ml), and the presence of particles of progressively decreasing size, causing a bimodal distribution of the lipoproteins between 1.015 and 1.085 g/ml, show the existence of IDL ($1.015 < \rho < 1.040$ g/ml) in the trout. These particles undoubtedly arise from the action of lipoprotein lipase of the vascular endothelium, highly activated by the VLDL in this species [23]. It has been shown in trout that LDL are distributed up to densities of 1.078–1.085 g/ml [2,3]. The use of density gradient centrifugation confirms that the cutoff density of 1.063 g/ml is insufficient for the LDL/HDL separation. This situation is similar to that encountered in chickens [24] guinea pigs [25], pigs [26] and chimpanzees [27]. The homogeneously sized LDL ($1.040 < \rho < 1.085$ g/ml) are more negatively charged than their human counterparts and have only one type of apoB-like at M_r 240000, as is the case for human LDL, which contain only apoB-100 [28,29]. The existence of an apoA-I-rich LDL fraction poses the problem of determining if this results from LDL enriched in apoA-I or the presence of a mixture of LDL and lipoproteins containing only apo A-I. Apolipoproteins A-I and A-II are the major protein constituents of HDL. The accumulation of M_r 55000, 76000 and apoA-like apolipoproteins between 1.182 and 1.234 g/ml suggests the presence of VHDL and/or free apolipoproteins. Concerning apolipoprotein M_r 55000, which is also a minor component of HDL, this behaviour is similar to that of human apoA-IV, which accumulates in the 1.21 g/ml infranatant [30–32].

The present work involved fractionating the plasma of female trout in previtellogenesis. Vitellogenin, whose monomer in SDS/glycerol/polyacrylamide-gel electrophoresis has an M_r of 175000 [9], is thus not observable. This lipoprotein, containing 18% lipids of which two-thirds are phospholipids [33] and which may be considered as a VHDL ($\rho > 1.21$ g/ml), is present specifically in egg-laying females during vitellogenesis. Yolk resorption by females in the course of massive intraovarian follicular atresia, in particular after ovulation, causes the appearance of egg yolk proteins in the plasma. These proteins are intimately associated with HDL and modify the apolipoprotein profile of this lipoprotein class [9].

Compared with intestinal absorption in mammals, that in fish is a slow process. The maximal absorption phase in trout occurs between 18 and 24 h [19,20] and the concentration of plasma lipids increases substantially during this phase in catfish [34]. After lipid intraluminal hydrolysis, the resulting dietary fatty acids are esterified in the intestinal epithelial cells [19,35] and this esterification leads to the formation of VLDL and chylomicrons [19–21]. The direct transfer of unesterified fatty acids into the circulation has also been reported [36–38]. This process seems to occur during early absorption. The unesterified fatty acids account for only a small proportion of fatty acids transported by the blood [5,19] and they are probably combined with plasma fatty acid binding proteins [39]. The concentration of plasma lipids in fish is highly dependent on their physiological and nutritional state. There currently exist few data concerning the quantitative variations of different lipoprotein classes. Sexual maturation in salmonids is accompanied by a reduction or total cessation of food intake [40]. In the pink salmon, this results in the presence of only HDL just before laying [8]. These variations in the levels of triacylglycerol-rich lipoproteins affect, for example, the activity of plasma lecithin:cholesterol acyltransferase (EC 2.3.1.43) [41,42]. These variations appear to also

exist in the trout, especially with age [7] or stage of sexual maturity [43].

The fractionation of trout plasma lipoproteins in a density gradient has led to the precise identification of different classes of lipoproteins, in particular as a result of the apolipoprotein profile. It is now possible to study all the factors contributing to the quantitative variations of these different lipoprotein classes.

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