Effects of 17β-Estradiol and Starvation on Trout Plasma Lipoproteins

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Administering 17β-estradiol (E_2) to juvenile trout results in plasma hyperlipidemia and hyperlipoproteinemia associated with significant increases in the concentrations of triglycerides (TG), free cholesterol, phospholipids, free fatty acids and proteins, both postprandial and during starvation. TG undergo the greatest increase (9 times control level 96 h after feeding). The concentration differences between E_2-treated and control trout increase during starvation, primarily by progressive decreases in the concentrations of various lipids in controls. E_2-induced hypertriglyceridemia is mainly caused by an increase in the concentration of very low density lipoproteins (VLDL) during both the postprandial period (6 times control level at 24 h) and during starvation (15 times control level at 96 h); hyperlipoproteinemia lasts up to at least 7 d after the last feeding. E_2 treatment does not change the concentration of high density lipoproteins, but does increase plasma concentrations of a very high density lipoprotein, vitellogenin (VTG). In E_2-treated VLDL, cholesteryl esters are depleted while proteins are enriched. During the postprandial phase, the apolipoprotein (apo) profile of VLDL (d < 1.015 g/mL) is comparable in E_2-treated and control trout. Starvation of E_2-treated trout is accompanied by an enrichment in apo B_{240}, A-I and A-II. The secretion levels of TG and VLDL-TG, as determined *in vivo* by injecting Triton WR-1339 to starving animals, are significantly higher in E_2-treated trout than in controls. In trout, as in chicks, E_2 administration significantly increases the concentration and hepatic secretion of plasma VLDL independent of the nutritional status and the appearance of VTG in the plasma. This suggests the existence of similar mechanisms for the regulation of lipoprotein metabolism by estrogens in oviparous vertebrates.
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Sex steroids modify the risk of atherosclerosis by regulating the levels of plasma lipoproteins (1–3). Natural or induced changes in estrogen concentrations in various species have been shown to affect the production and catabolism of lipoproteins. The mechanisms involved in the action of estrogens on lipoprotein metabolism appear to be largely dose dependent (4,5).

Pharmacological doses of estrogens in mammals stimulate the number of low density lipoprotein (LDL) receptors in rat (6,7) and rabbit liver (8) and in the HepG2 human hepatocarcinoma cell line (9). In humans, estrogens reduce hepatic triglyceride lipase activity (10–12) and increase the concentrations of plasma high density lipoproteins (HDL) (10–13) and very low density lipoproteins (VLDL) (11,14). In humans, estrogens also stimulate apolipoprotein synthesis by HepG2 cells (15,16). Plasma concentration and hepatic secretion of VLDL have been shown to increase in rats after treatment with ethinyl estradiol (17–19).

Vitellogenesis is characterized by the accumulation of large reserves of yolk by growing oocytes. Yolk proteins and lipids are derived from plasma precursors, primarily lipoproteins (20). Vitellogenesis in birds results essentially from oocyte uptake of two classes of different density lipoproteins, VLDL and vitellogenin (VTG), a very high density lipoprotein. Hepatic synthesis of these two macromolecular complexes is stimulated dramatically by estrogens during sexual maturation of females or during estrogen treatment of males or immature females (21,22). The molecular mechanisms of the regulation of lipoprotein and apolipoprotein syntheses have been studied in birds, especially in chicks (4,22,23). Estrogen-induced hyperlipidemia in this species is characterized by a pronounced increase in VLDL levels, a smaller increase in LDL levels and a decrease in HDL levels (24–26). VLDL possess only two major apolipoproteins (apo), apo B and apo VLDL-II; the latter is synthesized by the liver under the strict control of estrogens (22). The presence of apo VLDL-II results in a considerable decrease of VLDL sensitivity toward lipoprotein lipase (LPL) (27,28). These VLDL are then incorporated in growing oocytes by an apo B-specific surface receptor (29) which is identical to the VTG receptor (30).

VTG has been detected in the blood of a large number of fish species during their normal reproduction cycle or in response to estrogen stimulation (31). Its hepatic synthesis can be induced by 17β-estradiol (E_2) (32) and is correlated with the circulating levels of this hormone during vitellogenesis (33,34).

No data exist on the possible role of estrogens in the metabolic regulation of other lipoprotein classes in fish. The aim of this work was to investigate the combined effects of nutritional status and brief E_2 treatment in juvenile trout on the qualitative and quantitative changes in the different lipoprotein classes of the plasma. In particular, we have shown that in trout, as in chicks and rats, E_2 administration causes a significant increase in the concentration and secretion of plasma VLDL, independent of nutritional status.

MATERIALS AND METHODS

Plasma. Juvenile rainbow trout, *Oncorhynchus mykiss* (formerly *Salmo gairdneri*) were raised at constant temperature (13 ± 0.5°C) and nourished *ad libitum* three times a week with granules (Aqualim, Nersac, France) containing 8% lipids. The trout were anesthetized with ethylenglycol monophenyl ether (0.3 mL/L). Blood was removed over ethylenediaminetetraacetic acid (EDTA) disodium salt and NaN_3 (3 and 0.15 mg/mL blood) dissolved in 0.15 M NaCl, final pH 7.4, by cardiac punc-

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Abbreviations: Apo, apolipoprotein; BSA, bovine serum albumin; BW, body weight; CE, cholesteryl esters; E_2, 17β-estradiol; EDTA, ethylenediaminetetraacetic acids; FC, free cholesterol; FFA, free fatty acids; HDL, high density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; LW, liver weight; LPL, lipoprotein lipase; LSI, liver somatic index; PL, phospholipids; SDS, sodium dodecylsulfate; TC, total cholesterol; TG, triglycerides; TGSR, triglyceride secretion rates; VLDL, very low density lipoproteins; VTG, vitellogenin.

ture with a fine catheter (diameter 0.58 mm) and kept at 4°C throughout the procedure. Plasma was obtained by centrifugation (3000 × *g*, 10 min).

17β-Estradiol and fasting treatments. A graphic representation of our study design is shown in Figure 1. Animals were anesthetized and injected intraperitoneally with E_2 (7 mg/kg body wt) on days 0, 4, and 8; the injection volume was 1 μL/g body wt. E_2 was first dissolved in anhydrous ethanol and then in cottonseed oil (1:4, vol/vol). Controls were injected with ethanol and cottonseed oil without E_2. This treatment was selected to produce a rapid elevation in E_2 and VTG plasma levels, to the same range found during exogenous vitellogenesis in females (33–37). During the treatment there was no mortality, and the trout were still feeding.

Two-year-old juvenile trout of both sexes, weighing 278 ± 61 g (± SD, n = 26) from controls and the E_2-treated group were starved for 3 d and then fed *ad libitum* on day 10 of the treatment. The effects of starvation with or without estrogen treatment on plasma lipoprotein profiles were evaluated with serial blood samples taken until 7 d after the last meal. The blood volumes collected were lower than 2% of the whole body blood volume. Quantitative and chemical analysis of plasma lipids, proteins and lipoproteins in the two groups were performed with blood samples taken in series at 24, 96 and 154 h after feeding. The blood volumes punctured were lower than 10% of the total whole body blood volume. Alimentary assimilation is slower in fish than in mammals (31).

At the conclusion of the experiments (day 17, see Fig. 1) fasted fish were anesthetized and killed. Body (BW) and liver weights (LW) were determined to calculate the liver somatic index [LSI = (LW/BW) × 100].

Lipoprotein fractionation and analysis. A density gradient ultracentrifugation procedure previously described (38) was used with modifications for the fractionation of whole plasma lipoprotein classes and proteins. Discontinuous six-step density gradients were prepared with NaBr solutions, containing 0.05% EDTA (pH 7.4). Successive densities were (from top to bottom of tube): 1.006 g/mL (1.1 mL); 1.019 g/mL (2.5 mL); 1.063 g/mL (3.5 mL); 1.210 g/mL (2.5 mL); 1.310 g/mL (plasma at 1.310 g/mL, 0.55 or 0.01 mL, adjusted to this density with solid NaBr, and completed with a 1.310 g/mL NaBr solution to give a final volume of 1 mL); and 1.386 g/mL (0.8 mL). Six percent of the plasma volume is assumed to consist of macromolecules, and the remaining 94% is equivalent to a salt solution of density 1.015 g/mL. Instead of the adjusted plasma sample, control gradients were prepared using a NaBr solution of density 1.015 g/mL adjusted to 1.310 g/mL with solid NaBr. The gradients were then placed in a Beckman (Palo Alto, CA) SW 41-Ti swinging bucket rotor (average radius 110.2 mm) and centrifuged at 180,000 × *g* for 24 h at 10°C in a Beckman L8-70 ultracentrifuge, without braking at the end of the run.

The centrifuge tube containing the separated lipoproteins was punctured at the bottom and connected to the fractionation system filled with a 1.386 g/mL NaBr solution. The lipoprotein profile was recorded by continuously monitoring absorbance at 280 nm with a UV monitor (LKB 2238 Uvicord SII, LKB, Bromma, Sweden) as the lipoproteins were pumped from the centrifuge tube (collection speed 0.6 mL/min, chart speed 10 mm/min). One arbitrary unit of absorbance was taken as the optical

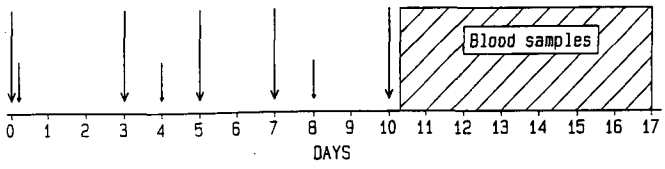

FIG. 1. Graphic representation of the study design. The small arrows indicate when 17β-estradiol or vehicle alone was intraperitoneally injected. The large arrows indicate when trout were nourished. The shaded area represents the period of blood sampling.

density of a 1 mg/mL solution of bovine serum albumin (BSA; Cohn fraction V, Sigma Chemical Co., St. Louis, MO). At the end of the fractionation, distilled water was carefully deposited onto the upper layer of the tube until VLDL was completely eluted. Fractions were collected with a fraction collector. Quantitative lipoprotein profiles were determined based on total lipoprotein concentration and the volume of the combined tube fractions (60 fractions, mean volume 300 μL). For each fraction, the lipoprotein concentration was calculated as follows: total lipoproteins = cholesteryl esters (CE) + free cholesterol (FC) + triglycerides (TG) + phospholipids (PL) + proteins. Density regions used were VLDL (VLDL + chylomicrons at + 24 h after feeding), d < 1.015 g/mL; LDL (intermediate density lipoproteins (IDL) + LDL), 1.015 < d < 1.085 g/mL; HDL, 1.085 < d 1.210 g/mL; VTG, (presence of VTG only for estrogen-treated fish), 1.210 < d < 1.310 g/mL; proteins, d > 1.310 g/mL. The concentrations were expressed as mg/dL plasma after correction for dilution introduced by the anticoagulant. The recovery after the entire procedure was 86.03 ± 4.32% for CE, 92.94 ± 2.93% for FC, 95.58 ± 3.24% for TG, 84.57 ± 3.09% for PL, and 88.73 ± 2.19% for proteins (n = 14).

The apolipoprotein compositions of the major classes of trout lipoproteins have recently been described (31,38, 39). Apolipoproteins were electrophoresed under reducing conditions in sodium dodecylsulfate (SDS)/glycerol/polyacrylamide slab gels using a linear gradient of 3.5–15% polyacrylamide and 8–12% glycerol (39). The relative molecular mass (Mr) values of trout apolipoproteins were determined as previously described (39) by comparison with simultaneously run proteins of known Mr (MW-SDS-70L and MW-SDS-200 molecular weight marker kits from Sigma Chemical Co.). Gels were subsequently fixed and stained using a highly sensitive Coomassie Blue G-250 procedure (40).

The size distribution of VLDL isolated 96 h after feeding was monitored by electron microscopy (80 kV) after dual staining of the particles (41). VLDL were diluted first with saline EDTA, mixed with 1:1 (vol/vol) 4% OsO_4, and fixed for 2 min. The fixed and positively stained particles were then negatively stained with 1% neutral phosphotungstic acid in the presence of 0.1% sucrose on a Formvar/carbon grid (Balzers, Selles S/Cher, France).

Chemical analysis. The lipid compositions of the lipoprotein fractions and of plasma were estimated using commercially available enzymatic kits from Boehringer-Mannheim (Meylan, France) for FC, total cholesterol (TC) and free fatty acids (FFA), and from Wako-Unipath (Dardilly, France) for PL and TG. The assays were performed as described elsewhere (42,43). Protein concentrations were determined by a modified Lowry assay (44) using BSA

as standard. The amount of CE was calculated using the formula CE = 1.7 × (TC−FC), using 288 as the average molecular weight of the trout plasma fatty acids (45). The amount of total lipids (TL) in plasma were calculated from the expression TL = CE + FC + PL + TG + FFA (46). Concentrations in plasma were expressed as mg/100 mL plasma after correction for the dilution introduced by the anticoagulant.

Ultrastructural study. The tissues were fixed for 1 h at 4°C in osmium tetroxide (OsO_4) buffered with sodium cacodylate (0.15 M, pH 7.3) and embedded in Epon 812. The lipids were preferentially contrasted by the OTO method (47). Thin sections of tissue were collected on gold grids and successively placed on 1% aqueous solution of thiocarbohydrazide (1 h at 50°C) and on 2% aqueous solution of osmium tetroxide (1 h at 50°C).

VLDL triglyceride secretion. To evaluate the effect of E_2 on TG and VLDL-TG secretion rates, trout were injected with Triton WR-1339 (Tyloxapol, Sigma Chemical Co.), a nonionic detergent which has been used widely as an indicator of VLDL secretion *in vivo* (25, 48–51). Eleven-month-old juvenile trout of both sexes, weighing 63.61 ± 10.3 g (±SD, n = 23) from controls and the E_2-treated group were starved for 96 h before Triton injection. Preliminary experiments indicated that adequate inhibition of TG removal occurs at 70 mg/100 g body wt or above. Animals were anesthetized, and Triton WR-1339 (25 g/dL in 0.9% NaCl, 70 mg/100 g body wt) was injected intracardialy at day 14 of the estrogen or control treatment (injection volume, 145 μL). For each trout, blood samples were taken intracardialy at 15 min (arbitrary zero time) and 3 h or 6 h after the Triton injection. The mean of TG secretion rates (TGSR) for each group, E_2-treated or control animals, was determined by the equation TGSR = 1/2 (TG1/T1 + TG2/T2) × PV (49). TG1 and TG2 are means augmentations of plasma TG concentrations (mg/dL) 3 and 6 h after Triton injection, and T1 and T2 are times between zero time and time of sampling after Triton injection. PV is the estimated whole body plasma volume (3.25 mL/100 g body wt) (52,53).

VLDL were isolated by micro-ultracentrifugation in an air-driven ultracentrifuge (Airfuge, Beckman, Palo Alto, CA) (54,55) from plasma of the same trout obtained 15 min (zero time) and 3 h after Triton injection. One hundred μL plasma was transferred to a centrifuge tube, layered gently with 75 μL of NaBr solution (d = 1.015 g/mL), and centrifuged at 100,000 rpm (30 psi) for 3 h in a fixed angle rotor (A-100/30). The air was refrigerated at 4°C using an air drying and cooling system (56). Thirty μL of top VLDL fraction was cut with a tube slicer specifically designed for the small tubes. TG values were determined in control and E_2-treated trout to evaluate the difference in VLDL output *in vivo* after Triton WR-1339 injection. Pure VLDL, not contaminated by other lipoprotein classes or plasma proteins, were isolated by this procedure (57). However, the volume collected here was too low for a complete recovery of the VLDL fraction (54,55).

Statistical methods. Data are presented as means ± SEM and were tested for statistical significance by analysis of variance followed by an evaluation employing Scheffe's multiple range test. LSI comparison was made by Student's *t*-test. Statistical evaluations of the results were performed using the Statistical Analysis System (SAS) (58). The *P* value chosen for statistical significance was 0.05.

RESULTS

Concentration of plasma lipid fractions and proteins. The effects of starvation on the concentration of different plasma lipids and proteins, with or without E_2 treatment, are shown in Table 1. Plasma samples were obtained from juvenile control or treated trout 24, 96 and 154 h after the last feeding.

E_2 treatment increased the concentrations of plasma lipids and proteins except CE, regardless of the time after feeding, with the levels of TG increasing the most. Plasma TG levels at 96 h were nine times higher in E_2-treated animals than in controls (874 mg/dL *vs.* 96 mg/dL). The other parameters assayed, FC, PL, FFA and protein, also increased in treated *vs.* control trout, reaching maxima at 154 h. Starvation in controls was accompanied by a significant decrease in the plasma concentrations of all lipids, except FC (total lipids = 1212 mg/dL at 24 h *vs.* 734 mg/dL at 154 h). In E_2-treated animals rendered hyperlipidemic and hyperproteinemic, only the plasma concentrations of CE and TG decreased during starvation (significant at $P < 0.05$ for TG), while those of PL and proteins increased significantly (PL = 802 mg/dL at 24 h *vs.* 1034 mg/dL at 154 h; proteins = 8019 mg/dL at 24 h *vs.* 11,879 mg/dL at 154 h). During fasting, triglyceridemia

TABLE 1

Changes in the Concentrations of Plasma Lipid Fractions and Proteins at Different Time Points After Feeding with and Without 17β-Estradiol Treatment in Juvenile Trout (mg/dL plasma)[a]

Time after feeding		Total cholesterol (mg/dL)	Free cholesterol (mg/dL)	Cholesteryl ester (mg/dL)	Triglyceride (mg/dL)	Phospholipid (mg/dL)	Free fatty acid (mEq/L)	Total lipids (mg/dL)	Protein (mg/dL)
+24 h	Control (9)	200 ± 27	75 ± 13	211 ± 25	358 ± 89	557 ± 66	0.335 ± 0.060	1212 ± 193	4840 ± 282
	Estradiol (9)	390 ± 50	235 ± 34[c]	262 ± 32	968 ± 28[d]	802 ± 55	0.526 ± 0.044	2323 ± 125[c]	8019 ± 410[d]
+96 h	Control (9)	179 ± 22	64 ± 8	194 ± 24	96 ± 17	437 ± 49	0.224 ± 0.044	799 ± 97	4750 ± 319
	Estradiol (8)	385 ± 43[b]	249 ± 30[d]	230 ± 31	874 ± 36[d]	922 ± 56[d]	0.580 ± 0.093	2291 ± 133[d]	9726 ± 440[d]
+154 h	Control (6)	164 ± 28	61 ± 9	175 ± 35	80 ± 20	416 ± 82	0.115 ± 0.017	734 ± 142	4582 ± 255
	Estradiol (4)	375 ± 45[b]	263 ± 28[d]	191 ± 31	675 ± 79[d]	1034 ± 51[d]	0.516 ± 0.170[b]	2178 ± 133[d]	11879 ± 269[d]

[a]Values are means ± SEM. Sample numbers are given in parentheses. At each time point after feeding, the significances for the differences between control and treated trout were tested using analysis of variance and Scheffe's multiple range test.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

in control trout dropped faster than in E_2-treated trout, but both were decreasing, thus amplifying the difference.

The increase in total plasma cholesterol in E_2-treated trout was not reflected in a concomitant increase in the plasma CE concentration. This indicates that the percentage of esterified cholesterol was lower in these animals and that the increase in cholesterolemia was due only to an increase in free cholesterol. The percentage of esterified cholesterol decreased even during starvation, from 63% in controls to only 30% after 154 h of starvation in E_2-treated animals.

Effects of starvation and 17β-estradiol on lipoprotein profile. The observed changes in plasma concentrations of the different lipids and proteins after feeding and E_2 administration revealed changes in plasma lipoprotein concentrations. A complete lipoprotein profile was obtained by fractionating 10 μL of plasma by density gradient ultracentrifugation. The lipoprotein profile was recorded by continuously monitoring absorbance at 280 nm as lipoproteins were pumped from the centrifuge tube. The different classes of lipoproteins were clearly identifiable (Fig. 2) and have been previously characterized (see ref. 38). Successive blood samples from the same animal, E_2-treated or control, illustrated the changes at different times after feeding.

The peak in alimentary absorption in controls was reflected in a substantial increase in the concentration of the TG-rich lipoproteins, VLDL and chylomicrons (d < 1.015 g/mL), reaching a maximum 16 h after feeding. At the time of the absorption peak and under standardized alimentary conditions, postprandial plasma triglyceridemia and the plasma concentration of the TG-rich lipo-

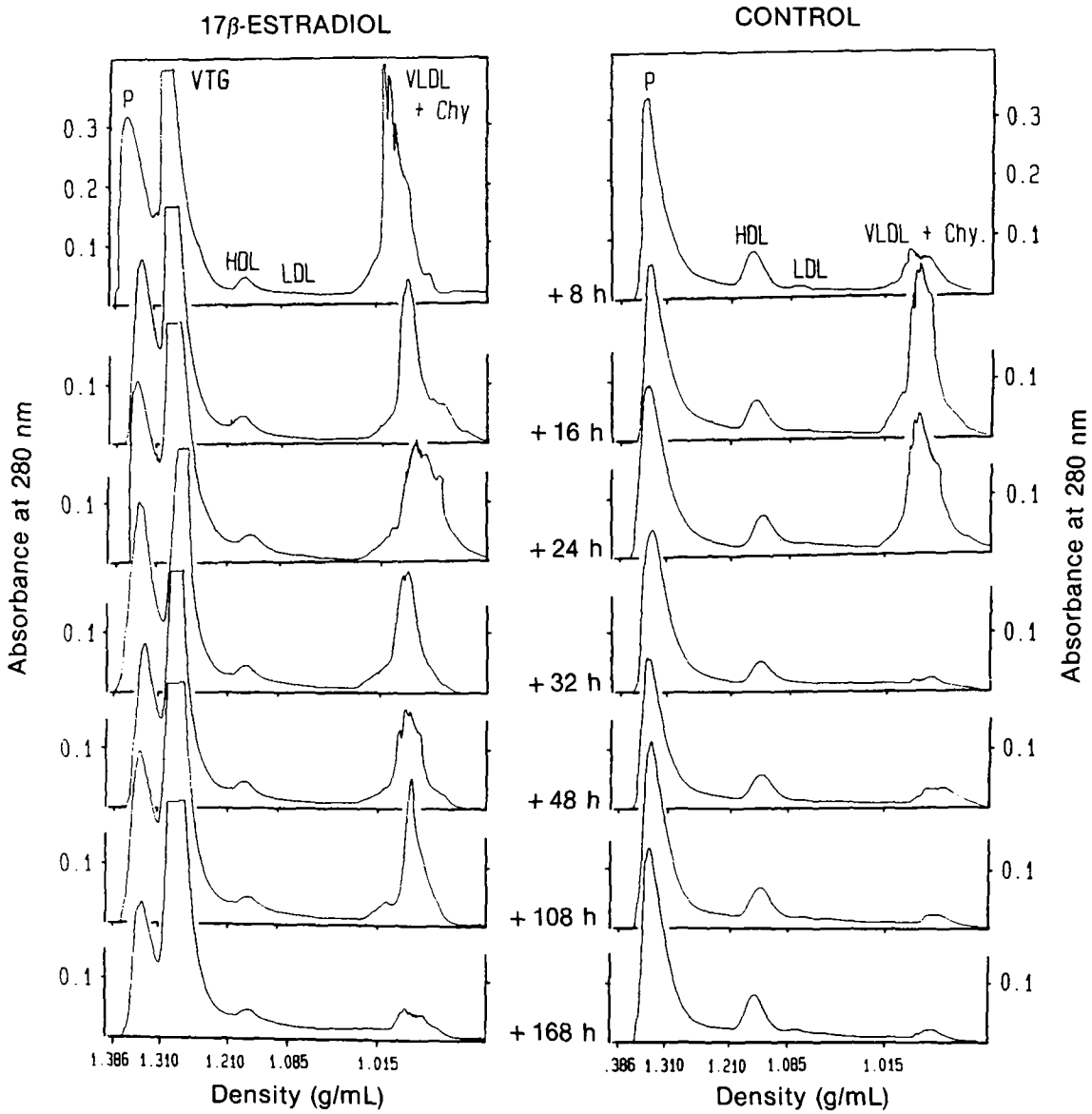

FIG. 2. Effects of starvation and 17β-estradiol on plasma lipoprotein profile in juvenile trout. Ultracentrifugal single-spin absorbance profiles of 10 μL of plasma were obtained at different hours after feeding. The two trout were selected for comparable postprandial triglyceridemia, 1126 mg/dL for the estrogen-treated trout *vs.* 1007 mg/dL for the control trout, 16 h after feeding. HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL + Chy., very low density lipoproteins + chylomicrons; VTG, vitellogenin.

proteins, VLDL and chylomicrons, depended on the quantity of food ingested during the meal (data not shown). To eliminate variations arising from nutritional variables, an E_2-treated trout and a control trout having comparable postprandial triglyceride levels and quantities of lipoproteins of $d < 1.015$ g/mL at the absorption peak were compared (Fig. 2) (TG 1126 *vs.* 1007 mg/dL at 16 h and 1108 *vs.* 957 mg/dL at 24 h; the lipoproteins were quantitated by integrating peak areas).

The administration of E_2 caused a very high concentration of VTG ($1.21 < d < 1.31$ g/mL) in the plasma. The concentrations of VLDL-chylomicrons and VLDL were much higher in E_2-treated trout than in controls. At 32 h, the quantity of circulating VLDL had returned to normal levels in controls (TG = 185 mg/dL), and it remained very high in E_2-treated trout (TG = 999 mg/dL), and lasted up to at least seven days of starvation (TG = 566 mg/dL in E_2-treated *vs.* 156 mg/dL in controls on day 7). The plasma concentration of VLDL-chylomicrons in E_2-treated trout was higher at 8 h than at the time when alimentary absorption peaked (16 and 24 h). The animals had been starved for 72 h (days 7 to 10) before the last feeding (see Fig. 1); therefore the peak at 8 h for the last feeding also represented the VLDL peak at 80 h (72 h + 8 h) for the penultimate feeding. The magnitudes of the VLDL peaks at 48 h and 108 h in Figure 2 clearly indicate that the peak at 8 h resulted from the addition of VLDL from the two feedings.

Concentrations and compositions of plasma lipoproteins. The quantitative changes in the different classes of lipoproteins in both groups of trout are listed in Table 2. Plasma hypertriglyceridemia induced by E_2 was due primarily to a significant increase in the concentration of VLDL, whether at the alimentary absorption peak (VLDL-chylo.: 6 × control level at 24 h) or in starving animals (VLDL: 15 × control level at 96 h). LDL (IDL + LDL), most of which are derived from lipolysis of VLDL, also were present in higher concentrations in E_2-treated animals than in controls, especially during starvation. Thus, 96 h after feeding, the mean concentration of LDL in treated animals was twice that in the controls (492 *vs.* 222 mg/dL), but the difference was not significant. E_2 treatment did not significantly change the concentrations of HDL and plasma proteins in the pellet ($d > 1.31$ g/mL). E_2 induced hepatic synthesis of VTG, a phospholipid-rich lipoprotein (18% lipids, of which 2/3 are phospholipids) which is the major protein in the plasma of E_2-treated trout. Due to the high density and the high concentration of VTG, it was not possible to satisfactorily separate VTG from the plasma proteins in the pellet (see Figs. 2 and 3). The values in Table 2 for juvenile control trout show the protein concentration in the density zone of 1.21 to 1.31 g/mL and do not imply the existence of VTG.

During starvation, the concentration of VLDL decreased in both control and E_2-treated trout, while LDL decreased in the controls (Table 2). The HDL concentration was not significantly changed in either controls or treated animals. The VTG concentration, however, increased significantly (twofold increase between 24 and 154 h), explaining the previously mentioned increase in plasma PL and proteins during starvation in E_2-treated trout (Table 1). The lipoprotein profiles presented in Figure 3 were typical of the mean concentrations induced (Table 2) after E_2 administration and/or starvation; they illustrate the increase in VLDL concentrations after E_2 administration (3492 *vs.* 973 mg/dL at 24 h, 1861 *vs.* 95 mg/dL at 96 h, 1046 mg/dL at 154 h). The progressive increase in VTG concentration during starvation also is well illustrated (3874 mg/dL at 24 h, 4772 mg/dL at 96 h and 8168 mg/dL at 154 h).

The percentages of lipid and protein in VLDL ($d < 1.015$ g/mL) of controls were different at 24 h and 96 h (TG/CE, 50:10, for core lipids and FC/PL/proteins, 5:23:12 for surface components at 24 h; *vs.* 45:14 and 6:23:12 at 96 h; in weight ratios). At 96 h, the core lipids were depleted in TG and enriched in CE. By contrast, the compositions of E_2-treated VLDL were identical at 24 h and 96 h (TG/CE, 49:6 for core lipids and FC/PL/proteins, 6:20:19, for surface components). However, the compositions of VLDL of E_2-treated trout and control trout were different. CE was depleted in E_2-treated VLDL, especially at 96 h, whereas protein was enriched. The increase in total plasma cholesterol in E_2-treated trout was not reflected in a concomitant increase in plasma CE concentration (Table 1). This was consistent with the high concentration of VLDL depleted in CE in E_2-treated trout.

A comparison of the VLDL apolipoprotein profiles of

TABLE 2

Effects of 17β-Estradiol Administration and Fasting on Plasma Lipoprotein Concentrations in Juvenile Trout (mg/dL plasma)[a]

Time after feeding		Bottom proteins (P)	Vitellogenin (VTG)	High density lipoproteins (HDL)	Low density lipoproteins (LDL)	Very low density lipoproteins (VLDL)
+24 h	Control	2405 ± 372(4)	1274 ± 233(4)	852 ± 172(5)	395 ± 122(5)	307 ± 158(5)
	Estradiol	1874 ± 123(7)	3836 ± 283(7)[b]	1117 ± 138(6)	547 ± 64(6)	2716 ± 50(7)[b]
+96 h	Control	2122 ± 352(4)	1052 ± 206(4)	838 ± 145(4)	222 ± 76(4)	118 ± 34(4)
	Estradiol	1494 ± 108(7)	5314 ± 368(7)[c]	1112 ± 108(7)	492 ± 32(7)	1861 ± 391(7)[b]
+154 h	Control	2017 ± 160(3)	901 ± 187(3)	657 ± 132(3)	73 (2)	189 (2)
	Estradiol	2372 ± 626(3)	6971 ± 976(3)[c]	1659 (2)	684 ± 131(3)	1179 ± 254(3)

[a]Values are means ± SEM. Sample numbers are given in parentheses. A density gradient ultracentrifugation procedure was used for the fractionation of whole plasma lipoproteins and proteins. The concentrations were calculated as follows: total lipoproteins = cholesteryl esters + free cholesterol + triglycerides + phospholipids + proteins. Density regions used were VLDL (VLDL + chylomicrons at + 24 h after feeding), $d < 1.015$ g/mL; LDL (LDL + IDL), $1.015 < d < 1.085$ g/mL; HDL, $1.085 < d < 1.210$ g/mL; VTG (presence of VTG only for estrogen-treated fish), $1.210 < d < 1.310$ g/mL; P (bottom proteins), $d > 1.310$ g/mL. At each time point after feeding, the significances of the differences between control and treated trout were tested using analysis of variance and Scheffe's multiple range test.

[b]$P < 0.05$.

[c]$P < 0.001$.

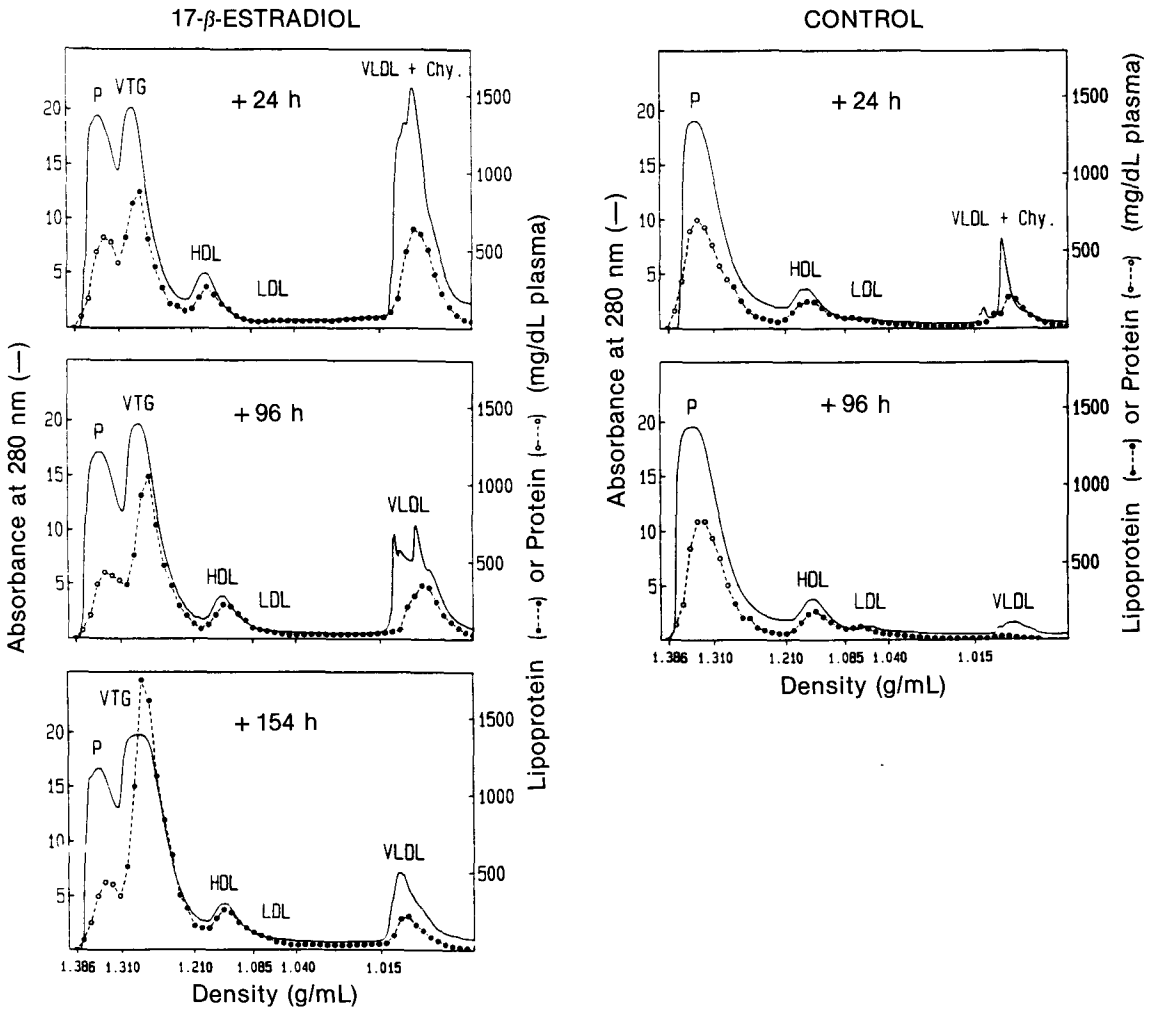

FIG. 3. Density gradient ultracentrifugal lipoprotein quantitative profiles of a 17β-estradiol (E_2)-treated or a control juvenile trout obtained at different times after feeding. The lipoprotein profiles presented were typical of the mean concentrations induced (Table 2) after E_2 administration and/or starvation. Absorbance at 280 nm (——), lipoprotein (when lipids were present in the fraction) (●—●—●), or protein (O—O—O) profiles. The lipoprotein concentration was calculated from the expression: total lipoproteins = cholesteryl esters + free cholesterol + triglycerides + phospholipids + proteins. The concentrations are expressed in mg/dL plasma. Abbreviations as in Figure 2.

control and E_2-treated trout did not show any new apolipoproteins in the E_2-treated animals (Fig. 4). At 24 h, the electrophoretic profiles were similar, but marked changes in the distribution of apolipoproteins were observed at 154 h. In control trout, prolonged starvation resulted in the total disappearance of Mr 240,000 apolipoprotein (apo B_{240}) which persisted in VLDL of E_2-treated trout. In these VLDL there was also a depletion of Mr 76,000 and 66,000 apolipoproteins at 154 h and an enrichment in species of Mr 25,000 (apo A-I) and Mr 13,000 (apo A-II).

The plasma concentration of VLDL in estrogenized trout was very high 96 h after feeding (Fig. 3). Electron microscopy of these VLDL (Fig. 5B) indicated that their size was homogeneous and that their mean diameter was lower than at the peak of alimentary absorption (24 nm *vs.* 20–50 nm) (31,38).

Ultrastructural study. The ultrastructural analysis of liver cells from estrogenized trout 96 h after feeding showed active synthesis of small VLDL, with a diameter similar to that observed in plasma (Fig. 5A and 5B). The cavities of the endoplasmic reticulum contained particles the size of VLDL which accumulated on the Golgi complex formation face. The extremities of the Golgi complexes formed secretion grains which released lipoproteins into intercellular space. The same feature was observed for control trout; however, at the end of the experiment, the LSI of E_2-treated and control animals were significantly different ($P < 0.001$): 4.01 ± 0.25% (n = 8) *vs.* 1.35 ± 0.11% (n = 9) and indicated hepatomegaly in the treated animals.

Triglyceride and VLDL-triglyceride output in vivo. The levels of plasma TG secreted by E_2-treated and control trout were determined *in vivo* after 96 h of starvation, after injecting Triton WR-1339.

Mean TG increases were 45.2 ± 14.6 mg/dL (n = 7) in E_2-treated animals *vs.* 15.1 ± 2.8 mg/dL (n = 8) in controls 3 h after injecting Triton, and 78.5 ± 34 mg/dL

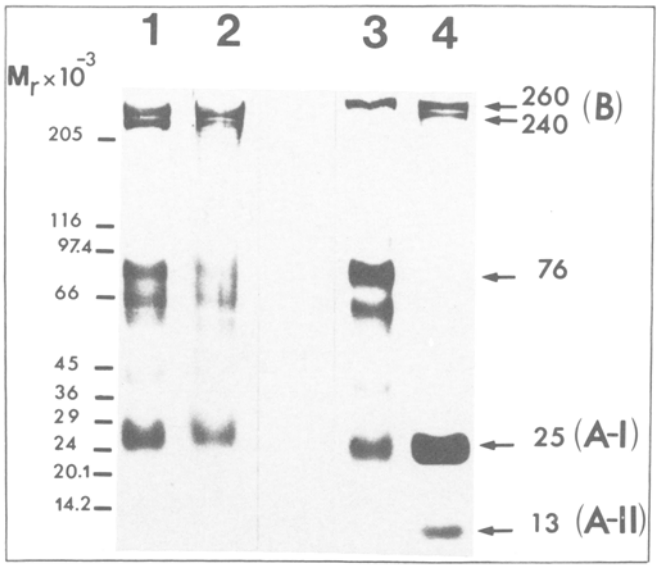

FIG. 4. Electrophoretic patterns in sodium dodecylsulfate/glycerol/polyacrylamide gradient gel of very low density lipoprotein (VLDL)-apolipoproteins from control and 17β-estradiol-treated trout (75 μg of proteins for lanes 1 and 2, 50 μg of proteins for lanes 3 and 4). 1, control VLDL 24 h after feeding; 2, 17β-estradiol-treated VLDL 24 h after feeding; 3, control VLDL 154 h after feeding; 4, 17β-estradiol-treated VLDL 154 h after feeding. Mr × 10^{-3}, reference markers relative molecular weight × 10^{-3}; (B), apo B; (A-I) apo A-I; (A-II), apo A-II (see references 31, 38 and 39 for details about trout apolipoproteins).

(n = 8) *vs.* 17.1 ± 3.2 mg/dL (n = 8) 6 h after injecting Triton. Based on these values the mean TGSR could be calculated as: 0.086 mg/h in controls and 0.308 mg/h in treated animals (control × 3.5) for a mean body of 63 g. The LSI values at the end of the experiment were significantly different ($P < 0.001$): 2.786 ± 0.23% (n = 11) in treated animals *vs.* 1.40 ± 0.06% (n = 11) in controls.

The difference in VLDL output, during starvation, between E_2-treated and control trout was determined by reproducibly isolating a part of plasma VLDL by microultracentrifugation 3 h after injecting Triton. Pure VLDL, not contaminated by other lipoprotein classes, were isolated. However, the volume collected was too small to completely recover the VLDL fraction. Under these conditions, the mean increases in VLDL-TG concentrations were significantly different: 17.1 ± 7.8 mg/dL (n = 5) in treated animals *vs.* 1.2 ± 0.4 mg/dL (n = 6) in controls.

DISCUSSION

Fish are poikilothermic vertebrates which preferentially utilize lipids rather than carbohydrates as energy source (59,60). Ovarian growth and reproduction activity are reflected by a clearcut increase in energy demand (61). There are two sources for this energy, one exogenous and of alimentary origin, the other endogenous, from lipid storage tissues (muscle, liver and possibly perivisceral adipose tissue) which are characterized by a succession of phases of deposition and mobilization of lipid reserves. Exogenous vitellogenesis, *i.e.*, endocytosis of the yolk precursors by oocytes, is shown by, among other things, elevated plasma concentrations of E_2 and testosterone, hypertrophy of the liver and the ovaries, and profound changes in lipid metabolism. These are reflected in qualitative and quantitative changes in the different classes of plasma lipoproteins.

Trout exhibit seasonal variations, which still are poorly defined, in the levels of the different classes of plasma lipoproteins other than VTG, and which are related to the annual reproductive cycle (31,62-64). In particular, the VLDL concentration increases during exogenous vitellogenesis (62,64). Studies on the effects of sex steroids on plasma lipids unrelated to VTG have been few in fish (65,66). No data exist on the possible role of sex hormones in the metabolic regulation of various classes of plasma lipoproteins other than VTG.

The injection of E_2 into juvenile trout enabled us to obtain LSI and VTG concentrations comparable to those in adult trout in exogenous vitellogenesis (31,67). Our results show that in addition to inducing VTG synthesis, E_2 led to a considerable increase in the concentration and secretion of VLDL in plasma. As in chicks (68) and rats (18), the increased concentration of VLDL appears to be due to stimulated hepatic secretion of VLDL. These effects on VLDL are apparently common to all vertebrates (11,14,17,19), particularly oviparous (24-26,68). Induced hypertriglyceridemia in trout arises primarily from the increased concentration of the triglyceride-rich VLDL and, to a lesser extent, LDL, whereas VTG in this species contains only 4% TG (69). In fish, as in mammals, most LDL are derived from the sequential lipolysis of VLDL to IDL and then LDL (38,70), which would explain the slightly increased concentration of LDL in E_2-treated trout.

Hyperlipidemia and hyperproteinemia induced by E_2 in trout are at least partially due to increased secretion of lipoproteins, which persists after several days of starvation, consistent with recent observations in chicks (71). The increased levels of FFA under the effect of estrogens, which are especially pronounced during starvation in trout (as shown here), but also in chicks (71,72) and lizards (73), suggest that estrogens also may cause the mobilization of fatty acids in reserve tissues (71,73-75).

Alimentary assimilation is slower in fish than in mammals (31). The appearance of TG-rich lipoproteins in plasma, characteristic of the postprandial absorption peak, thus occurs later in trout than in humans. After the postprandial phase, progressive decreases in VLDL and LDL concentrations were observed, while HDL levels stayed the same, in agreement with other observations in trout during starvation (76). The very high concentrations of VLDL in E_2-treated animals also were associated with a slow and gradual decrease in VLDL concentrations during starvation, indicating that plasma clearance is effective and/or hepatic secretion is reduced. The progressive increase in VTG concentration during starvation may be due to VTG accumulation in plasma of juvenile trout, in the absence of ovarian uptake.

In chicks, the VLDL present in plasma during E_2 treatment are very different from the VLDL of controls, especially in size, electrophoretic migration and apolipoprotein composition (77). The VLDL of E_2-treated chicks are composed of at least two different lipoprotein classes with different particle sizes and apolipoprotein compositions. Small VLDL were predominant in E_2-treated chicks (24,77).

In fish, as in other vertebrates, the peak of alimentary absorption results in a mixture of TG-rich lipoproteins of

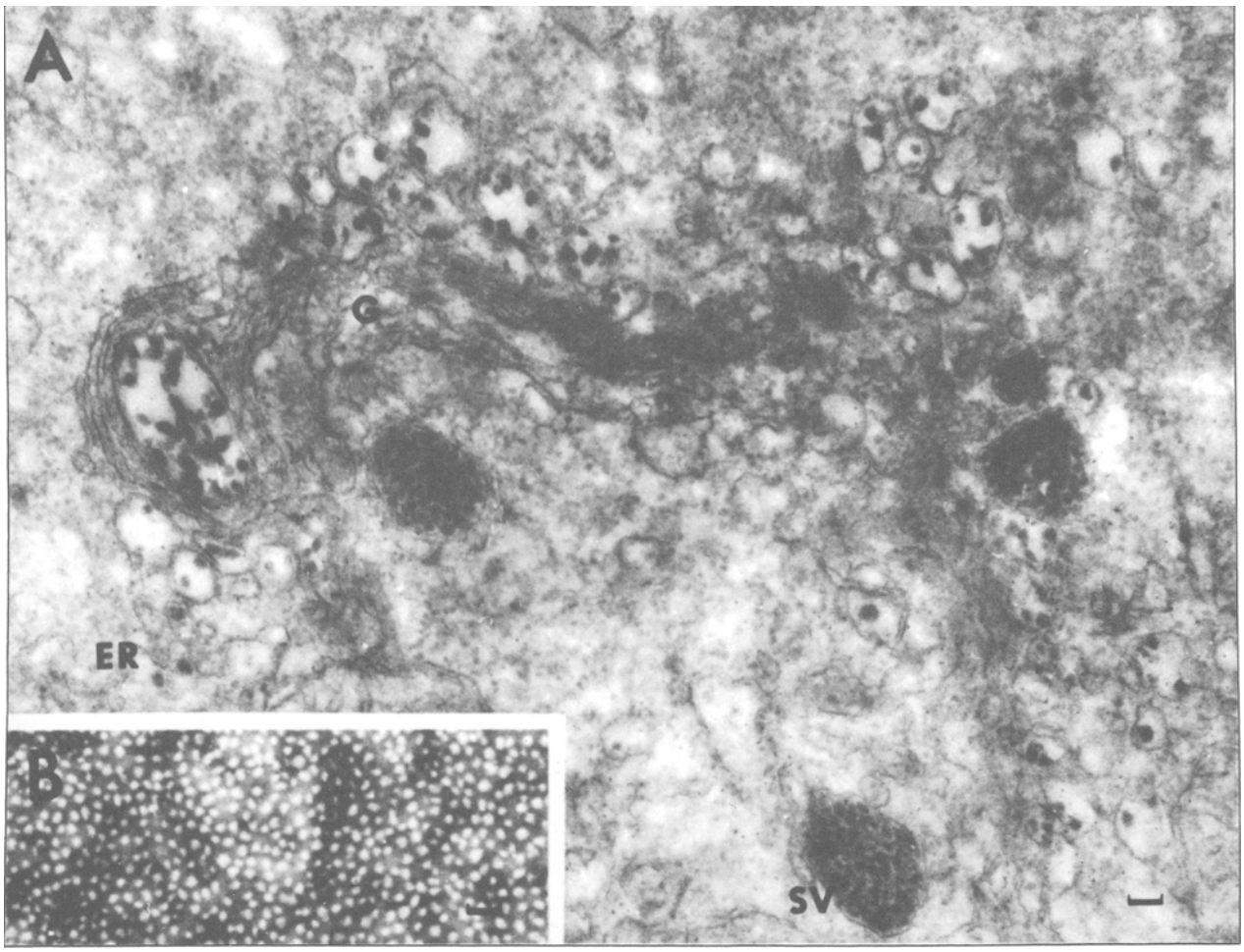

FIG. 5. Liver cell ultrastructure (A) and plasma VLDL (B) of juvenile estrogenized trout in the course of fasting (96 h after feeding). (A), ultrastructural analysis was carried out on sections in which lipids were contrasted by the OTO method. Very low density lipoproteins appeared as black particles in endoplasmic reticulum (ER), Golgi complex (G), and secretory vesicles (SV). (B), lipoproteins were isolated by density gradient ultracentrifugation (d < 1.015 g/mL), positively stained with OsO_4, and then negatively stained with phosphotungstate. Scale bar = 100 nm.

intestinal (78,79) and hepatic origin (79) in the plasma. Fasting results in a progressive disappearance of TG-rich intestinal lipoproteins (78). In E_2-treated animals, there is a high concentration of small VLDL in the plasma, similar to those synthesized by the liver, and being enriched in certain apolipoproteins. These results suggest that treatment with E_2 may change the apolipoprotein distribution of endogenous hepatic VLDL.

The increased rate of secretion of TG or VLDL-TG after injecting Triton WR-1339 in fasting animals, between control and estrogenized trout, may be partially due to the increase in the LSI. This index, increased by a factor of two or three in estrogenized trout, indicates hepatomegaly in these animals. Hepatomegaly is coupled with active synthesis of VLDL by liver cells and may be responsible for the very high concentration of plasma VLDL in estrogenized fish. The increase in net secretion of hepatic VLDL does not exclude the possibility of a simultaneous decrease in the activities of plasma lipases or in the production of VLDL particles, which are less sensitive to the action of LPL. Aside from the modified CE and protein contents of the entire VLDL pool and an enrichment of the fraction in certain apolipoproteins during starvation, we were unable to show qualitative differences in the apolipoprotein composition of VLDL between E_2-treated and control trout (38,39). In chicks, the presence of apo VLDL-II in VLDL after E_2 treatment is reflected in a decrease in their sensitivity to LPL (27,28) and by slower catabolism of the particles in the plasma of males (77). In laying hens, this decreased sensitivity toward LPL enables these VLDL to be incorporated in growing oocytes (80), mediated by an apo B-specific surface receptor (29) which is identical to the VTG receptor (30). Even though no direct proof exists, the strategy appears to be different in trout. In addition to taking up VTG (81) by a specific surface receptor (82), the ovary may be able to take up lipid components by lipolysis of circulating lipoproteins. This is suggested by the considerable increase in LPL and salt-resistant lipase activities in the ovary during exogenous vitellogenesis (63). LPL in this species is greatly activated by VLDL and, to a lesser extent, by HDL (83).

In conclusion, estrogen-induced hyperlipidemia in the juvenile trout results in major changes in lipoprotein profiles. Estrogen-induced changes in the profiles can occur spontaneously in females during the reproductive cycle. The increased concentration of VLDL and the appearance

of VTG after administering E_2 are similar to those observed in chicks and suggest the existence of similar mechanisms for the regulation of lipoprotein metabolism by estrogens in oviparous vertebrates.

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