Synthesis of very low density lipoproteins in the cockerel. Effects of estrogen.

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Research Article

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Synthesis of Very Low Density Lipoproteins in the Cockerel

EFFECTS OF ESTROGEN

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ABSTRACT The effect of estrogen on the synthesis of plasma very low density lipoproteins (VLDL) in the cockerel was studied both in vivo and in vitro. Synthesis was studied by immunoprecipitation techniques with antisera prepared against VLDL and a major VLDL protein. VLDL were isolated from the plasma of white Leghorn hens and estrogen-treated white Leghorn cockerels by ultracentrifugal flotation at *d* 1.006 g/ml. After delipidation, the lipid-free proteins (apoproteins) were fractionated on Sephadex G-150 and DEAE-cellulose. Both the hen and the estrogen-treated cockerel VLDL were shown to contain an identical apoprotein with a mol wt of approximately 12,000; the apoprotein is designated fraction B. Reduction and S-carboxy-methylation of fraction B resulted in a reduction of the molecular weight by approximately one-half, indicating a dimer-monomer relationship. Antiserum prepared to the hen VLDL dimer protein gave precipitin lines of complete identity to both the hen and cockerel dimer, monomer, VLDL, apoVLDL, low density lipoproteins, and plasma; no precipitin line was formed with either hen or cockerel high density lipoproteins.

After a single subcutaneous injection of diethylstilbestrol into the cockerel, plasma VLDL protein, cholesterol, and triglyceride increased, reaching a maximum 24–48 h after hormone administration. Liver slices from similarly treated animals were incubated in vitro in culture medium in the presence of [^{3}H]lysine for 2 h. Immunoprecipitable radioactivity in VLDL increased within 2 h of diethylstilbestrol treatment and reached a maximum at 24 h; VLDL radioactivity returned to base-line levels by 72 h. At the peak of induction, newly synthesized VLDL represented 11% of the total soluble protein synthesized. When actinomycin-D (5 mg/kg) was administered simultaneously with estrogen, the induction of VLDL synthesis was totally inhibited.

To determine whether the effect of estrogen on VLDL synthesis was mediated at the level of transcription, partially-purified cockerel liver mRNA was prepared from estrogen-treated animals and the mRNA activity for fraction B was quantitated in a wheat germ translation system. Fraction B mRNA was found to increase from a low base-line value to a maximum 16–24 h after estrogen treatment, returning towards base-line values at 30 h. At the peak of induction, fraction B constituted 12% of the total protein synthesized. The kinetics of induction of fraction B mRNA activity in the cell-free translation system is very similar to that observed in liver slice experiments. This finding suggests that estrogen stimulates VLDL synthesis, at least partially, by enhancing the accumulation of the mRNA for one of their major apoproteins.

INTRODUCTION

It is now well known that multiple genetic and environmental factors modulate lipoprotein metabolism (1). For example, estrogen is one factor which profoundly influences plasma triglyceride levels (2, 3). In the amphibian *Xenopus laevis,* estrogen induces the synthesis of a major yolk lipoprotein, lipovitellin (4).

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Similarly, in avian species, lipovitellin synthesis is stimulated (5, 6) and concomitantly the level of immunoprecipitable lipovitellin-synthesizing polysomes is increased (7). Hillyard et al. (8) demonstrated that in white Leghorn cockerels, estrogen caused a dramatic increase in the level of plasma very low density lipoproteins (VLDL).[1] This effect of estrogen was shown by Luskey et al. (9) to be mediated, at least in part, by an increased rate of synthesis of the lipoproteins.

Because of the relationship of estrogen to hypertriglyceridemia (2, 10, 11), we have undertaken a study to investigate the mechanism of the induction of VLDL synthesis by estrogen. In the present communication, we report a study of the rate of induction of VLDL by estrogen in the cockerel. The mRNA for a major apo-VLDL was partially purified from the cockerel liver and translated in a heterologous cell-free system. Moreover, estrogen was shown to regulate the levels of translatable mRNA for apoVLDL in a manner which suggests an effect at the level of gene transcription.

METHODS

Animals. 3-wk-old white Leghorn cockerels (0.2 kg) were used in the study. Induction of VLDL synthesis in the cockerel was studied after the animals were given a single subcutaneous injection of diethylstilbestrol (DES) 2.5 mg in sesame oil. This dose of DES was maximally effective in inducing VLDL synthesis. The animals were killed at appropriate times by cervical dislocation or decapitation. In experiments involving actinomycin-D, the drug was dissolved in water (1 mg/ml) and injected intramuscularly (5 mg/kg). All animals were fed a standard chicken diet and were housed in a room that was lighted daily from 7 a.m. to 7 p.m. For the preparation of large amounts of lipoproteins, plasma from white Leghorn laying hens (2 kg) was obtained commercially (Pel-Freeze Bio-Animals, Inc., Rogers, Ark.). All blood was collected in 0.1% EDTA–0.01% sodium azide, pH 7.5.

Isolation of lipoprotein and apoproteins. VLDL ($d <$ 1.006 g/ml), low density lipoproteins (LDL) (d 1.006–1.063), and high density lipoproteins (HDL) (d 1.063–1.210) were isolated by ultracentrifugal flotation procedures (12) in KBr at 8°C in a Beckman model L2-65B preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Each centrifugation was performed for 20 h at 55,000 rpm in a 60 Tirotor. The isolated VLDL fraction was recentrifuged twice at d 1.006 g/ml. After lyophilization, the dry lipoprotein (5 mg) was delipidated by extracting (30 ml) twice with diethyl ether-ethanol (3:1) at 4°C followed by two washes with diethyl ether; the lipid-free proteins (apoVLDL) contained less than 1% phospholipid by weight as determined by the method of Bartlett (13). ApoVLDL were solubilized in 0.1 M Tris-HCl, pH 8.2, containing 0.1 M sodium decyl sulfate and were fractionated on Sephadex G-150 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) and DEAE-cellulose by procedures described previously for human apoVLDL (14–16). LDL were subjected one time to recentrifugation at d 1.063 g/ml and the reisolated lipoproteins dialyzed overnight against 0.01% EDTA, pH 7.5. HDL were isolated as described previously for human HDL (17).

The purity of the isolated fractions was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (18); by amino acid analysis and the absence of certain residues; and by immunochemical methods using goat antiserum prepared against the purified apoprotein. Tryptophan was determined by the method of Liu and Chang (19).

Quantitative determination of plasma VLDL proteins and lipid content. Animals in groups of four were treated with DES as described above. The animals were decapitated and blood was collected in 0.1% EDTA, pH 7.5. Erythrocytes were removed by low speed centrifugation. VLDL were isolated by ultracentrifugation at plasma density (12) and were dialyzed overnight against 0.01% EDTA, pH 7.5. Protein was determined by the method of Lowry et al. (20). Total cholesterol and triglycerides were determined by autoanalyzer techniques (21).

Preparation of antisera against VLDL and apoVLDL and determination of equivalence point. Antisera against VLDL were prepared by injection into the foot pads of rabbits whereas antisera against a major VLDL protein (fraction B) were prepared by subcutaneous injection of the purified apoprotein into a goat every 2–3 wk. Animals were bled and the globulin fraction purified by ammonium sulfate precipitation (0–50%). After dialysis against 0.01 M Tris-HCl, pH 8.0, the antisera were heated at 56°C for 30 min, centrifuged at 12,000 rpm at 4°C for 30 min to remove the insoluble material and stored at −20°C until used.

To define the conditions for optimal precipitation of VLDL or apoVLDL by the antisera, increasing amounts of VLDL or apoprotein were added to a constant amount of antiserum. The incubation mixture contained 5% Triton X-100, 1% sodium deoxycholate, and 10 mM sodium phosphate, pH 7.5. Incubation was at 23°C for 3 h or at 23°C for 30 min and then overnight (16 h) at 4°C. Both methods were found to give maximal precipitation. Immunoprecipitates were collected by centrifugation at 2,000 g for 5 min. The supernatant fluid was decanted and the precipitate was washed three times, first with a standard wash buffer containing 10 mM sodium phosphate, pH 7.5, 15 mM sodium chloride, 4 mM L-lysine, 1% sodium deoxycholate, and 1% Triton X-100 followed by two washes with 0.9% NaCl. The protein content of the washed pellet was determined by the method of Lowry et al. (20).

In vitro induction of VLDL synthesis. The right lobe of the liver was removed and was cut into 50–100-mg pieces. The tissue was transferred to 25-ml Erlenmeyer flasks (200–350 mg/flask) and incubated in 2 ml of medium 199 without amino acids (Grand Island Biological Co., Grand Island, N. Y.); one-eighth the usual amount of amino acids present in medium 199 was added. The medium also contained 1.2 mg/ml of $NaHCO_3$, 1 U/ml of penicillin and 1 mg/ml of streptomycin. 25 μCi of L-4,5-[^{3}H]-lysine monohydrochloride (sp. act. 19 Ci/mmol, Amersham-Searle Corp., Arlington Heights, Ill.) was then added. The tissue was incubated at 37°C with continuous shaking in the presence of 95% O_2–5% CO_2. With these incubation conditions, incorporation of L-[^{3}H]-lysine into immunoprecipitable and trichloroacetic acid (TCA) precipitable material was linear for at least 2 h. After incubation, excess (0.5 mmol) cold

[1] *Abbreviations used in this paper:* apoVLDL, lipid-free proteins; DES, diethylstilbestrol; HDL, high density lipoproteins; LDL, low density lipoproteins; S-30, 30,000 g supernatant fluid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; VLDL, very low density lipoproteins.

L-lysine was added, the tissues were weighed, and homogenized in the incubation medium by six strokes in a glass-Teflon homogenizer. The homogenate was then centrifuged at 105,000 *g* for 60 min at 4°C. The supernatant fluid was used for determination of radioactive VLDL and TCA-precipitable radioactivity.

Quantitation of in vitro synthesized VLDL. Aliquots from the 105,000 *g* supernate were incubated with a rabbit anti-VLDL globulin fraction in the presence of carrier VLDL at a concentration to precipitate 50% of the antiserum. A typical incubation mixture consisted of the following: sample 200 μl, antisera 75 μl, carrier VLDL (1 mg/ml) 5 μl, 20% Triton X-100 400 μl, 10% sodium deoxycholate 100 μl, 100 mM sodium phosphate, pH 7.5, 50 μl, and 100 mM L-lysine 5 μl. Incubation was at 23°C for 3 h or at 23°C for 30 min, and then overnight at 4°C. Immunoprecipitates were collected by centrifugation at 2,000 *g* for 5 min. The supernatant fluid was decanted and the immunoprecipitate was washed three times, once with wash buffer and twice with 0.9% NaCl. The washed pellet was dissolved in 1 ml of NCS (Amersham-Searle Corp.) and counted in 10 ml of spectrofluortoluene. The nonspecific radioactivity was determined by precipitating the supernatant fluid a second time and subtracting this amount of radioactivity from the amount precipitated in the first immunoprecipitate. Usually, 150–300 cpm were nonspecifically trapped, whereas the cpm in the first precipitate ranged from 700 to 7,000. VLDL synthesized in vitro were also quantitated by ultracentrifugation. The 105,000 *g* supernate obtained from the in vitro liver slice incubation was dialyzed extensively against six changes of 0.9% NaCl, 0.01% EDTA, pH 7.5, at 4°C. The dialyzate was then centrifuged in a Beckman SW 50.1 rotor at 50,000 rpm for 18 h at 8°C; the VLDL were removed (~0.7 ml) and aliquots counted in spectrofluor-Triton X-100.

Isolation of partially purified liver mRNA. Total liver nucleic acid was prepared from DES-injected cockerels by the method of Rosenfeld et al. (22). Liver tissue from five cockerels was pooled and homogenized in a Waring blender (Waring Products, New Hartford, Conn.) at 23°C in 5 vol (vol/wt) of a sodium dodecyl sulfate (SDS) buffer containing 0.5% SDS, 25 mM EDTA, 75 mM NaCl, pH 8.0, and an equal volume of phenol saturated with the SDS buffer and adjusted to pH 8.0 with NaOH. The aqueous phase was separated by low speed centrifugation, removed and re-extracted with the phenol. SDS was precipitated by cooling the mixture on ice and was removed by centrifugation. Total nucleic acid in the aqueous phase was precipitated in 2 vol of 95% ethanol at −20°C in the presence of 0.2 M NaCl.

Polyadenylated RNA in the total RNA preparation was selectively adsorbed on nitrocellulous membrane filters (Millipore Corp., Bedford, Mass.) as described by Rosenfeld et al. (22). The RNA was applied to the filters at 23°C in the presence of 0.5 M KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.6, and eluted from the filters with 0.5% SDS in 0.1 M Tris-HCl, pH 9.0 (22). When mRNA activity was assayed in the wheat germ system as described below, the millipore adsorption method was found to give a 5-fold purification of total mRNA activity. In general, from each gram of liver, about 5–7 mg of total nucleic acid was recovered by the extraction procedure, and about 1% of the total RNA was recovered from nitrocellulose membrane filters.

In vitro protein synthesis in the wheat germ system. In vitro protein synthesis was carried out using a 30,000 *g* supernatant fluid (S-30). The S-30 fraction was prepared from wheat germ by the method of Roberts and Paterson (23) as modified by Means et al. (24). The wheat germ was kindly supplied as a gift by General Mills, Inc., Minneapolis, Minn., was ground to a fine powder before the buffer was added, and the S-30 fraction was desalted on a column of Sephadex G-25. The components of the system were as follows: 24 mM Hepes, pH 7.6, 2 mM dithiothreitol, 1 mM ATP, 20 μM guanosise triphosphate, 8 mM creatine phosphate (Sigma Chemical Co., St. Louis, Mo.), 400 μg/ml creatine phosphokinase, 84 mM KCl, 2.5 mM Mg $(C_2H_3O_2)_2$, 20 μM each of unlabeled amino acids except leucine, 4 μM L-[U-^{14}C]-leucine (320 mCi/mmol), an appropriate amount of RNA, and 20 μl of wheat germ S-30. The total volume of the mixture was 100 μl. Incubation was carried out at 25°C for the time indicated in the Figure Legends. At the end of incubation, the mixture was centrifuged at 105,000 *g* for 60 min. Aliquots were taken from the supernatant fluid for determination of TCA precipitable radioactivity and for quantitation of VLDL by immunoprecipitation. For TCA precipitable radioactivity, the in vitro product was suspended in 10% TCA and heated for 10 min. The precipitate was collected on glass-fiber filters, washed with cold 5% TCA, and the radioactivity adsorbed to the filter was determined by liquid scintillation spectrophotometry in spectrofluor-Triton X-100.

Quantitation of immunoprecipitable apoVLDL synthesized in vitro. The in vitro synthesized fraction B was precipitated by goat anti-fraction B serum and sufficient fraction B to effect 50% precipitation of the antiserum. A typical reaction mixture contained the following: S-30 in vitro product 100 μl, 20% Triton X-100 50 μl, 5% sodium deoxycholate 100 μl, fraction B antisera 75 μl, and carrier fraction B (0.5 mg/ml) 5 μl. After 3 h incubation at 23°C, the reaction mixture was layered onto a solution containing 400 μl of 1 M sucrose in 10 mM leucine, 1% Triton X-100, and 1% sodium deoxycholate and the solution was centrifuged at 8,000 *g* in 1.5-ml polypropylene tubes in a Brinkman centrifuge (model 3200) (Brinkman Instruments, Inc., Westbury, N. Y.) for 5 min at 23°C. The supernatant fluid was aspirated and the immunoprecipitate was washed once with wash buffer. The washed pellet was dissolved in NCS and counted in spectrofluor-toluene.

Sodium dodecyl sulfate acrylamide gel electrophoresis of fraction B synthesized in vitro. The washed immunoprecipitate was analyzed by a modification of the procedure as described by Weber and Osborn (18). The precipitate was solubilized by incubation at 90°C in the presence of 50 μl of 1% SDS, and 10 mM dithiothreitol for 5 min. The solution was cooled and glycerol was added to given a final concentration of 10%; 10 μl bromophenol blue was added as a marker dye. The entire mixture was then applied to 15% polyacrylamide gels (12 cm), and the samples were subjected to electrophoresis at 3 mA per gel for 4–6 h at 23°C. Authentic fraction B was treated similarly and analyzed simultaneously with the immunoprecipitate. Gels were stained at 23°C with 0.2% Coomassie Blue in 50% methanol and 7% acetic acid. Destaining was accomplished by soaking the gels in 5% methanol and 7% acetic acid. After electrophoresis, radioactive gels were frozen on dry ice and cut into slices of about 2 mm thick using a razor blade slicer. The gel slices were solubilized by incubation overnight at 60°C in 500 μl 30% hydrogen peroxide and counted in 5 ml Aquasol (New England Nuclear, Boston, Mass.). The nonradioactive stained gels were scanned at 600 nm in a Gilford spectrophotometer gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

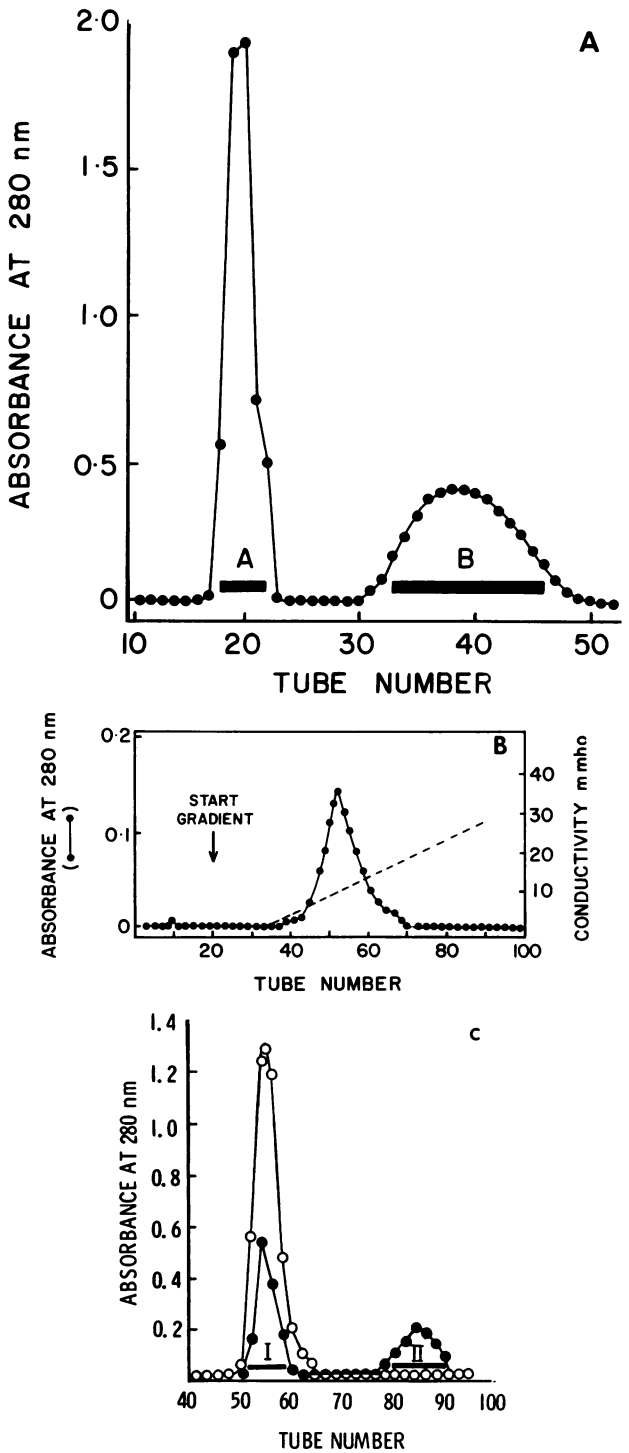

FIGURE 1 (A) Gel filtration of hen apoVLDL (32 mg) on Sephadex G-150. The column (1.6 × 200 cm) was equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM sodium decyl sulfate and 0.01% EDTA. After application of the sample, the flow rate was adjusted to 25 ml/h and 5-ml fractions were collected.
(B) DEAE-cellulose chromatography of peak B from Sephadex G-150 (Fig. 1A). The column (1.6 × 50 cm) was equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 6.0 M urea and was operated at room temperature. The sample (18 mg) was applied to the column and eluted with the equilibrating buffer. At tube number 20, the column was eluted with a NaCl gradient. To one side of the two-chambered gradient apparatus were added 300 ml of the equilibrating buffer and to the other side 300 ml of the same buffer, but containing 0.125 M NaCl. The flow rate was adjusted to 25 ml/h and 5-ml fractions were collected.
(C) Chromatography of fraction B and reduced S-carboxymethylated fraction B on Sephadex G-75. The column (1.6 × 200 cm) was equilibrated with 0.1 M Tris-HCl, pH 8.0, containing 6.0 M urea. Fraction B (-○-○-) or reduced S-carboxymethylated fraction B (-●-●-), 20 mg each, were applied to the column and eluted at a flow rate of 20 ml/h; 5-ml fractions were collected.

RESULTS

Isolation and characterization of hen plasma apoVLDL. VLDL were isolated from hen plasma by ultracentrifugal flotation at plasma density. The VLDL were delipidated and the lipid-free proteins were fractionated by gel filtration chromatography on Sephadex G-150 (Fig. 1A); two major protein-containing peaks were detected. Fraction A eluted at the void volume of the column and represented 52% of apoVLDL. Fraction B represented 43% of the total protein and eluted from the column as if it were a small molecular weight protein. Polyacrylamide gel electrophoresis of Fractions A and B are shown in Fig. 2. Fraction A did not enter the gel indicating that it was aggregated or had a high molecular weight. Based on its behavior on Sephadex and gel electrophoresis (Fig. 2B), fraction A resembled the major apoprotein from human VLDL (16) designated apoB. Fraction A was not further characterized in the present study. Fraction B (subsequently referred to as fraction B) contained a major apoprotein that migrated on polyacrylamide gel electrophoresis in SDS as if it had a mol wt of approximately 12–13,000 (Fig. 2C).

Chromatography of fraction B on DEAE-cellulose (Fig. 1B) gave a single protein-containing peak that represented 98% of the protein applied to the column. Polyacrylamide gel electrophoresis of this fraction is shown in Fig. 2D; one major band was detected. In the presence of the reducing agent dithiothreitol, there was a reduction in the mol wt of the apoprotein from about 12,000 to approximately 6,000 (Fig. 2E). Chromatography of fraction B on Sephadex G-75 in urea gave a single protein peak (Fig. 1C). Reduction and S-carboxymethylation of fraction B followed by chromatography on Sephadex G-75 is shown in Fig. 1C; two protein-containing peaks were detected. With the exception of S-carboxymethylcysteine in fraction II, both fractions had identical amino acid compositions. Further

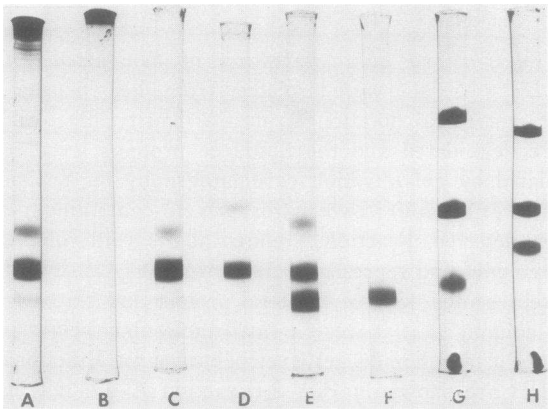

FIGURE 2 Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of hen apoVLDL and isolated apoproteins. 10–20 μg of protein were applied to the gels. A. ApoVLDL; B. Fraction A (Fig. 1A); C. Fraction B (Fig. 1A); D. DEAE fraction (Fig. 1B); E. DEAE fraction plus dithiothreitol; F. Reduced S-carboxymethylated fraction B; G. Standard proteins plus dithiothreitol; H. Standard proteins. The standard gels consisted of the following: top, bovine albumin (mol wt of 67,000); middle, human apoA-I (mol wt of 28,313); and bottom, human apoA-II (mol wt of 17,400) in gel H or (8,700) in gel G. The gels were stained with Coomassie brilliant blue.

reduction, alkylation, and rechromatography of fraction I yield more fraction II. The gel electrophoresis pattern for the alkylated derivative (fraction II, Fig. 1C) is shown in Fig. 2F. Based on its migration, reduced-alkylated fraction B has a mol wt of approximately 6,000.

Characterization of fraction B. Goat antiserum prepared against hen fraction B formed single precipitin lines of complete identity between fraction B, apoVLDL, and VLDL (Fig. 3A). However, no precipitin line was formed against Sephadex fraction A. The antiserum also gave identical precipitin lines (Fig. 3B) with hen LDL (*d* 1.006–1.063 g/ml), and plasma, but none were detected with hen HDL (*d* 1.063–1.210 g/ml). The amino acid compositions of Sephadex-fraction A and fraction B are given in Table I. The most noticeable difference between the two fractions was the absence of histidine in fraction B. Based on a monomer mol. wt of 6,000, fraction B contained 51 amino acid residues with 1 residue each of proline, cysteine, methionine, and phenylalanine.

Comparison of fraction B from the hen and the estrogen-treated cockerel. A single cockerel was given an injection of estrogen as described in Methods. 18 h later, blood was collected and VLDL were isolated from the plasma as described for the hen. Chromatography of cockerel apoVLDL on Sephadex G-150 and DEAE-cellulose gave elution profiles (not shown) that were identical to those obtained for hen apoVLDL. Fraction B from the cockerel formed single precipitin lines of complete identity against anti-hen fraction B (Fig. 3B). Furthermore, the lines were identical to those for hen fraction B, either as the dimer or the reduced-alkylated derivative. As in the hen, cockerel VLDL, LDL, and plasma but not HDL reacted with antiserum against hen fraction B (Fig. 3B). There was also a faint precipitin lines against cockerel LDL and plasma which is thought to be due to partial delipidation during ultracentrifugation. Amino acid analysis of cockerel fraction B was identical to that of fraction B from the hen (Table I).

Plasma VLDL in control and estrogen-treated cockerels. The concentration of VLDL in 3-wk-old untreated cockerels is very low, as determined by ultracentrifugal flotation of plasma at *d* 1.006. After a single injection of DES (2.5 mg), there was a slight, but always reproducible, decrease in VLDL concentration that occurred within 2 h of injection (Fig. 4). The initial fall was followed by an increase in plasma VLDL. The maximum amount of plasma VLDL occurred 24 and 48 h after DES injection. The accumulation of VLDL protein seemed to precede that of VLDL cholesterol and triglyceride suggesting that

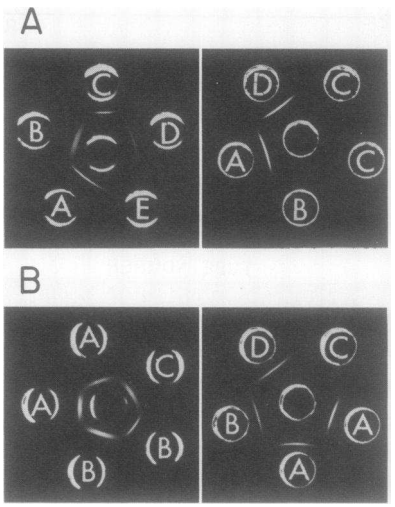

FIGURE 3 (A) Immunodiffusion of hen lipoproteins and apoproteins. The center wells contain 0.01 ml of goat antibody prepared against the DEAE-fraction (Fig. 1B). Each outer well contained approximately 10 μg in 0.01 ml of the indicated antigens. Left: A. Sephadex fraction B (Fig. 1A); B. DEAE fraction (Fig. 1B); C. Hen VLDL; D. Hen apoVLDL; E. Sephadex fraction A (Fig. 1). Right: A. Hen VLDL; B. Hen HDL; C. Hen LDL; D. Hen plasma. (B) Immunodiffusion of ten and cockerel lipoproteins and apolipoproteins. Each center well contained 0.01 ml of goat antibody prepared against the DEAE-fraction (Fig. 1B). Each outer well contained approximately 10 μg in 0.01 ml of the indicated antigens. Left: A. Cockerel fraction B; B. Hen fraction B; C. Reduced S-carboxymethylated hen fraction B. Right: A. Cockerel VLDL; B. Cockerel LDL; C. Cockerel HDL; D. Cockerel plasma.

VLDL protein synthesis is an earlier event, compared to the other components found in VLDL. The VLDL protein increased about 5-fold at the peak, whereas VLDL cholesterol increased some 25-fold and triglyceride some 7-fold. 72 h after estrogen treatment, the protein and lipid constituents had returned to control values.

Equivalence assay for anti-VLDL and anti-apoVLDL. To determine the optimal conditions for maximal precipitation of VLDL or fraction B by the antisera, increasing amounts of VLDL or fraction B were added to a constant amount of anti-VLDL or anti-fraction B. As shown in Figs. 5A and 5B, the equivalence point was 2 μg for VLDL protein and 12.5 μg for fraction B. Beyond these amounts, additional VLDL or fraction B resulted in partial solubilization of the immunoprecipitate. To ensure maximal precipitation of VLDL synthesized in the liver slices and the wheat germ extract, VLDL or fraction B were added in subsequent assays to ensure 50% of the maximal amount of total protein precipitated.

VLDL synthesis in liver slices in vitro. The synthesis of VLDL by liver slices incubated in vitro with L-[^{3}H]lysine is shown in Fig. 6A. Maximum synthesis of VLDL occurred at 24 h after DES, and it represented a 6-fold increase over control animals. The rate of synthesis declined in 48 h to control values. Animals injected with sesame seed oil alone did not show any significant increase in VLDL synthesis. The effects of DES on total protein synthesis is very erratic, and even control animals injected with oil alone showed minor changes (Fig. 6B). This increase may be related to the stress elicited by the handling of the animals as the changes were most observable within 2 h of injection. In general, DES-injected animals showed a 2-fold increase in the rate of total protein synthesis which lasted for about 36 h.

To exclude the possibility that the observed induction of VLDL synthesis by DES might be part of a general

TABLE I

Amino Acid Composition of Hen and Cockerel Apolipoproteins

Amino acid	Hen fraction A*	Hen fraction B‡	Rooster fraction B§	Hillyard et al. (27)‖
Lysine	8.13	2.93 (3)	3.38	3.65
Histidine	1.55	—	—	0.58
Arginine	3.96	3.85 (4)	3.71	2.38
Tryptophan	ND	0.40¶ (1)	ND	0.93
Aspartic acid	11.25	5.26 (5)	5.02	5.67
Threonine	6.67	3.18 (3)	3.23	3.26
Serine	8.36	2.80 (3)	2.89	3.69
Glutamic acid	12.09	6.00 (6)	5.71	5.88
Proline	3.88	0.94 (1)	1.07	1.88
Glycine	4.65	2.32 (2)	2.57	2.31
Alanine	6.38	5.03 (5)	5.10	3.83
Cysteine	ND	0.56 (1)**	—	0.51
Valine	4.69	3.96 (4)	3.71	3.43
Methionine	2.96	0.52 (1)	0.57	1.05
Isoleucine	5.41	2.50 (3)	2.90	3.40
Leucine	10.86	5.99 (6)	5.74	5.41
Tyrosine	3.76	2.16 (2)	2.18	1.77
Phenylalanine	5.28	1.18 (1)	1.18	2.21
Total	100	51		
mol wt		5938		

The values represent duplicate analyses on samples for 24 h.
* Based on 100 mol recovered. Fraction A is the protein shown in Fig. 1A.
‡ Based on mol wt 6,000. Hen fraction B represents the protein recovered from DEAE-cellulose chromatography (Fig. 1B). The number in parentheses refers to assumed values.
§ Based on 51 residues. Rooster fraction B represents the protein recovered from Sephadex G-150 and DEAE-cellulose as shown for the hen protein in Fig. 1.
‖ Recalculated based on 51 residues.
¶ Determined by the method of Liu and Chang (19).
** Determined as S-carboxymethylcysteine.

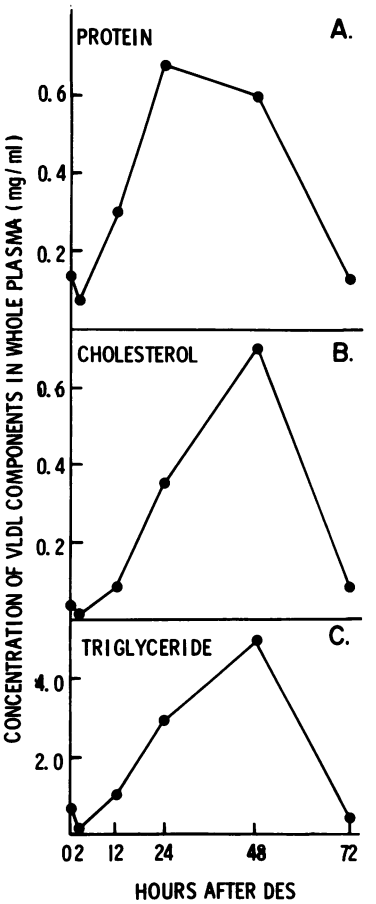

FIGURE 4 Effects of estrogen on plasma VLDL protein and lipids. Groups of 4 cockerels were treated with a single injection of DES (2.5 mg) subcutaneously. Animals were decapitated at the indicated times, and VLDL were isolated as described in Methods. Protein, cholesterol, and triglyceride contents in the purified VLDL were determined as described in Methods.

phenomenon of the effect of the hormone on total protein synthesis in the liver, the immunoprecipitable counts in VLDL were divided by the TCA-precipitable counts and this fraction was plotted vs. the time after DES treatment (Fig. 6C). From this plot, VLDL synthesis

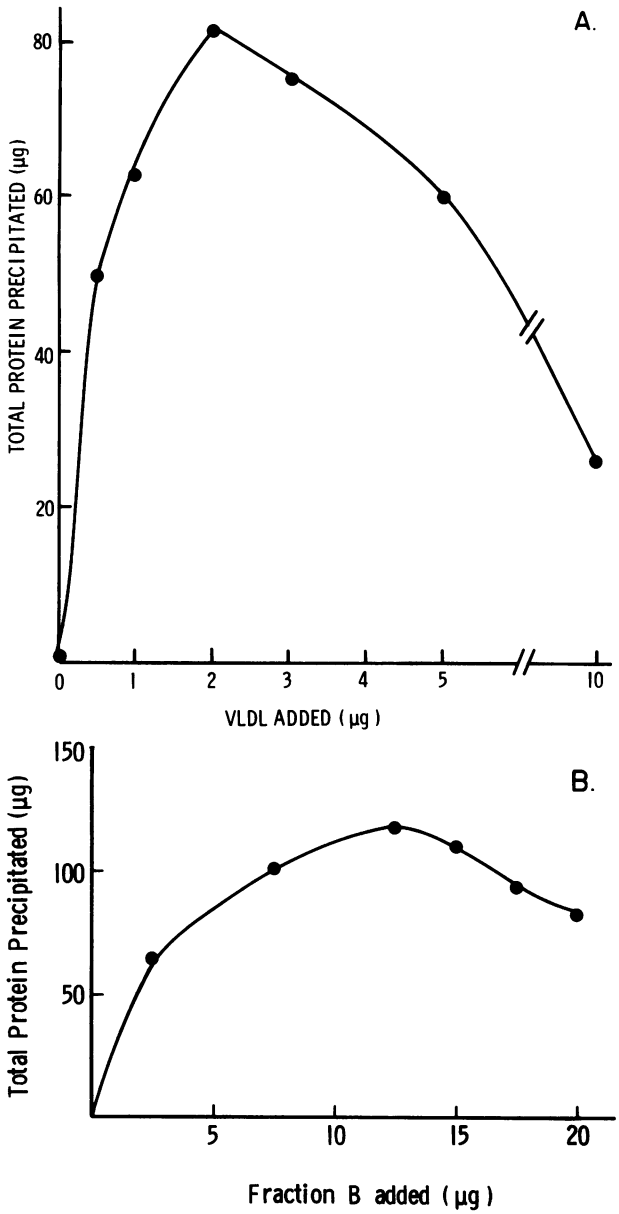

FIGURE 5 Determination of equivalence for anti-VLDL and anti-fraction B.
(A) Increasing amounts of VLDL were added to 75 µl of rabbit anti-VLDL. Incubation, washing, and protein determination on the precipitates were carried out as described in Methods.
(B) Increasing amounts of fraction B added to 25 µl goat anti-fraction B. Incubation, washing, and protein determination on the precipitates were carried out as described in Methods.

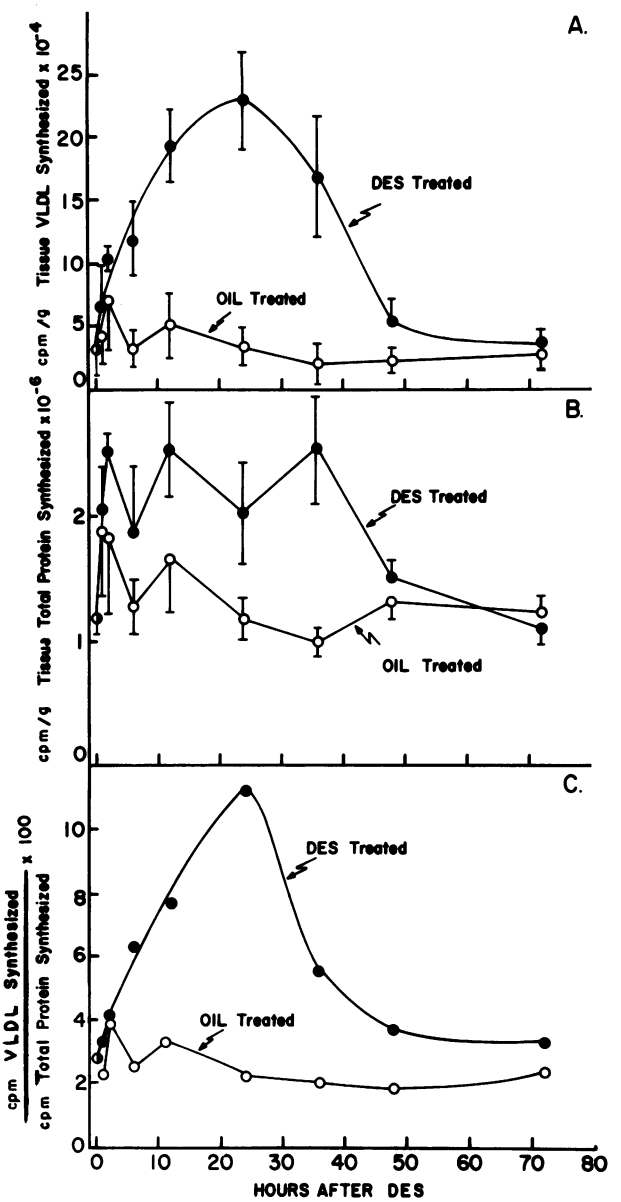

FIGURE 6 Effects of DES on protein and VLDL synthesis in liver slices. Groups of four cockerels were treated with a single subcutaneous injection of either DES (2.5 mg) or sesame oil. At the indicated times after injection, liver slices were incubated in vitro as described in Methods.
(A) Immunoprecipitable VLDL were determined on the liver homogenates as described in Methods. Values are $\bar{x} \pm SD$.
(B) Total TCA-precipitable counts were determined and taken as the total protein synthesized. (-●-●-), cpm in liver homogenate of DES-treated animals. (-○-○-), cpm in liver homogenate of oil-treated control animals. Values are $\bar{x} \pm SD$.
(C) VLDL synthesized as proportion of total protein synthesized was obtained by division of the values of 3A by those from 3B. (-●-●-), ratio in DES-treated animals. (-○-○-), ratio in oil-treated controls.

peaked at 24 h and returned to basal values by 48 h; control animals did not show any significant changes. At the peak of induction, VLDL constituted approximately 11% of the total soluble protein synthesized by the liver slices.

To further confirm that the labeled immunoprecipitable VLDL indeed represented newly synthesized VLDL, VLDL were isolated by ultracentrifugal flotation from the same samples as those used for immunoprecipitation. Unlabeled carrier VLDL were added to each sample before centrifugation. The ratio of counts in VLDL after estrogen treatment to the counts present before hormone treatment was calculated and used for comparison of the two methods. As indicated in Table II, the ratios were similar at 2, 24, and 48 h after estrogen treatment.

Effect of actinomycin-D on the estrogenic induction of VLDL synthesis. The effect of inhibition of RNA synthesis on the estrogenic induction of VLDL was studied using actinomycin-D which was given (5 mg/kg) by intramuscular injection to 3-wk-old cockerels simultaneously with 2.5 mg DES subcutaneously. Cockerels treated with DES alone served as the controls. As shown in Table III, actinomycin-D totally inhibited the increase in VLDL synthesis. These results suggest that RNA synthesis is required for induction of VLDL by estrogen. Time points later than 6 h were not done because actinomycin-D-treated animals became too sick to give meaningful data.

Fraction B synthesis in wheat germ in vitro. In Fig. 7A is depicted the time course of incorporation of [^{14}C]leucine into TCA precipitable material and immunoprecipitable fraction B; the incorporation was linear for approximately 1 h (Fig. 7B). The translation system was saturated when 4 μg of liver mRNA was added to the assay mixture (Fig. 7A). Control experiments without added mRNA gave only background counts which did not significantly increase with time. Additional control experiments (Table IV) using mRNA preparations from rat testis gave comparable

TABLE II

*VLDL Synthesis in Liver Slices as Determined by Immunoprecipitation and Ultracentrifugation**

Time after DES	cpm VLDL after DES/cpm VLDL at 0 h	
	By immunoprecipitation‡	By ultracentrifugation§
h		
2	3.16	2.65
24	6.97	6.90
48	1.67	1.63

* White Leghorn cockerels were given a single subcutaneous injection of 2.5 mg of DES. At the indicated times, the animals were sacrificed, and VLDL synthesis was determined as described in Methods.

‡ Immunoprecipitation was carried out as described in Methods. The ratios were derived from Fig. 6.

§ Four ~2-ml samples (105,000*g* supernatant fractions, aliquots of which were used for immunoprecipitation) were pooled together and dialyzed extensively against six changes of 0.01% EDTA, pH 7.4, over the course of 4 days. VLDL were isolated by ultracentrifugal flotation as described in the Methods. Aliquots of the VLDL were counted in Aquasol.

TCA-precipitable counts but anti-fraction B immunoprecipitable counts were not different from background. Similarly, when carrier ovalbumin was precipitated by an ovalbumin antiserum in the wheat germ assay in the presence of liver mRNA, radioactivity trapped in the immunoprecipitate was not significantly different from that trapped in the absence of added mRNA.

Sodium dodecyl sulfate acrylamide gel electrophoresis of apoVLDL synthesized in vitro. To further characterize the apoprotein synthesized in the wheat germ system, the immunoprecipitable material was analyzed by SDS-polyacrylamide gel electrophoresis. For this purpose, a 500-μl assay was used, with proportionate increases in all the assay components; the assay mixture was incubated at 25°C for 60 min. When the immunoprecipitate was solubilized and analyzed on the gel, the

TABLE III

*Effect of Actinomycin-D on Estrogen-Induced VLDL Synthesis**

Treatment	10^{-6} × cpm total protein‡	10^{-4} × cpm VLDL§
None (n = 3)	1.07±0.05	3.64±1.10
6 h after DES (n = 3)	2.17±0.23 ($P < 0.005$)	13.55±1.15 ($P < 0.005$)
6 h after DES + actinomycin-D (n = 3)	1.14±0.13 ($P > 0.05$)	3.98±0.94 ($P > 0.05$)

* Actinomycin-D (5 mg/kg) was given intramuscularly simultaneously with DES (2.5 mg) subcutaneously.

‡ cpm total protein refers to TCA precipitable cpm per gram of liver tissue. The values are expressed as the $\bar{x}$±SEM.

§ cpm VLDL refers to cpm in newly synthesized VLDL by immunoprecipitation per gram of liver tissue. The values are expressed as the $\bar{x}$±SEM. The P values are as determined by the Student t test.

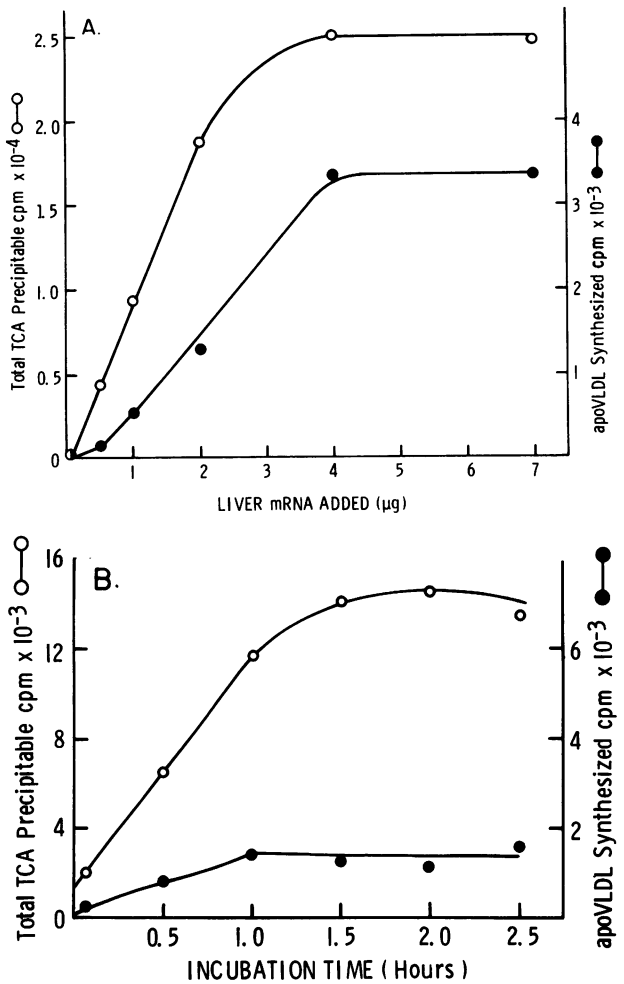

FIGURE 7 In vitro fraction B synthesis in wheat germ. The wheat germ translation system was prepared by a modification of the method of Roberts and Paterson (23). Partially purified cockerel liver mRNA was prepared as described in Methods.
(A) Incubation was at 25°C for 60 min. TCA precipitable counts and counts in immunoprecipitable fraction B were determined as in Methods.
(B) 2 μg liver mRNA was added to each assay and incubation carried out at 25°C for different time periods.

radioactivity was found to co-migrate with nonradioactive native fraction B analyzed simultaneously on another gel (Fig. 8). Thus, the peptide synthesized in vitro was not only similar immunochemically but was also found to have the same size as the authentic fraction B.

Effects of estrogen on fraction B mRNA levels. In Fig. 6, it was shown that estrogen increased the rate of synthesis of VLDL in cockerel liver slices in vitro in those animals that had been given the hormone in vivo. An increased rate of synthesis of fraction B of apoVLDL could have resulted from an increase in the

TABLE IV
*Tissue Specificity of Fraction B Synthesized In Vitro**

Messenger RNA added	cpm
Liver mRNA‡ (1 μg)	
TCA§	5,432
Fraction B antiserum‖	516.9
Ovalbumin antiserum¶	22.4
Rat testis mRNA** (2 μg)	
TCA§	5,836
ApoVLDL antiserum‖	18.4

* Translation assays were carried out using the wheat germ system as described in Methods.
‡ Liver mRNA was isolated from cockerel liver 24 h after a single injection of DES as described in Methods.
§ Background (no mRNA added) of 1,100 was subtracted.
‖ Background (no mRNA added) of 220 was subtracted. Fraction B antiserum precipitation was carried out as described in Methods.
¶ Background (no mRNA added) of 97 was subtracted. Ovalbumin antiserum precipitation of added ovalbumin carrier was carried out as described by Chan et al. (30).
** Rat testis mRNA was isolated from mature rat testis by the same method used for isolation of liver mRNA as described in Methods.

level of the mRNA coding for the apolipoproteins or from an activation of preexisting inactive mRNA molecules as has been suggested by Tomkins et al. (25). To differentiate between these two possible mechanisms, mRNA fractions were prepared from cockerel livers at various times after a single subcutaneous dose of DES and were translated in the wheat germ as described above. As shown in Fig. 9, there was an increase in

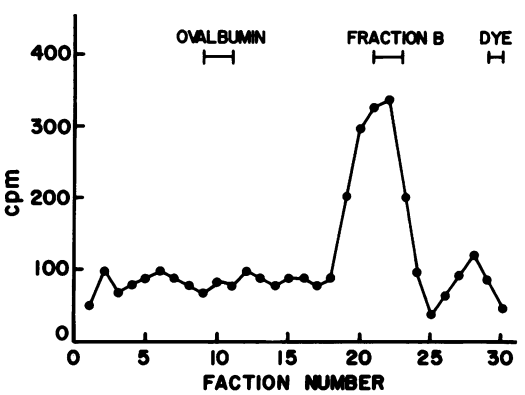

FIGURE 8 SDS-gel electrophoresis of apoVLDL synthesized in vitro. Fraction B synthesized in vitro and precipitated by fraction B antiserum was analyzed on 15% acrylamide gel electrophoresis as described in Methods. 2-mm slices were made and counted in Aquasol after solubilization in 30% H_2O_2. Parallel runs were made of fraction B and ovalbumin and scanned at 600 nm in a Gilford spectrophotometer gel scanner.

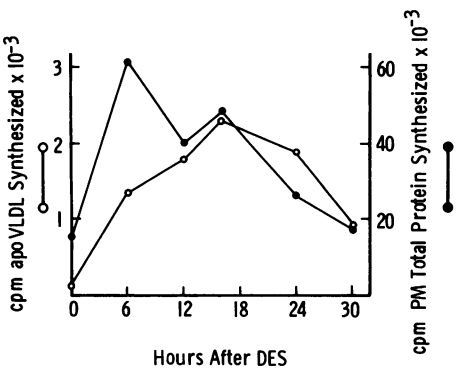

FIGURE 9 Effect of estrogen on fraction B mRNA activity. Total mRNA was prepared from cockerel liver at different times after one subcutaneous injection of DES (2.5 mg) as described in Methods. In vitro translation was carried out in the wheat germ system with subsaturating amounts of mRNA (less than 3 μg mRNA/100 μl assay). Total protein synthesized as well as fraction B synthesized were determined as described in Methods. All the cpm in the figure are expressed as activity per μg of mRNA added.

TCA precipitable counts in 6 h which remained elevated for about 16 h, and returned to base-line levels at 30 h. When immunoprecipitable fraction B was determined, a somewhat different curve was obtained. The total fraction B mRNA activity increased from a low basal level to a peak at 16–24 h, declining toward basal level at 30 h. The curve was similar to the induction curves obtained in liver slice incubation in vitro, where a maximal increase in rate of VLDL synthesis occurred at 24 h after DES. An even more striking similarity in the two situations is shown in Fig. 10 where the ratio of radioactivity in immunoprecipitable fraction B to that in TCA precipitable material is plotted as a function of time after DES treatment. The fraction B synthesized in vitro constituted an increasing percentage of total protein synthesized in the cell-free system with time after hormone administration, from a low basal level of 1% to a peak of about 12% at 24 h.

DISCUSSION

The effect of estrogen on plasma VLDL in the chicken was first noted by Hillyard and co-workers (8). This observation was subsequently confirmed and extended by others (9, 26). In a recent study, Luskey et al. (9) showed that estrogen stimulated the synthesis of VLDL in the rooster liver. These authors isolated the VLDL from the plasma of estrogen-treated roosters by ultracentrifugal flotation. Using antisera against VLDL, they demonstrated that there was a 4-fold increase in the rate of hepatic synthesis of VLDL antigen 16 h after an intramuscular injection of 60 mg of 17-β-estradiol. In the present report, we have examined the effects of estrogen on VLDL synthesis and accumulation both in vivo and in organ culture. We have also purified a major apoprotein in VLDL, fraction B, and have prepared specific antisera against this apoprotein.

Based on immunochemical and amino acid analyses, our studies show that VLDL from both the hen and the estrogen-treated rooster contain fraction B. By SDS-gel electrophoresis, the apoprotein consists of two polypeptide chains of 6,000 mol wt. The two chains are linked by a single disulfide bond. Hillyard et al. (27) also showed that hen VLDL contained a small molecular weight component. However, since they did not fractionate the VLDL, it may be assumed that their preparation contained both fractions A and B. The latter authors did not compare hen and cockerel VLDL. The previous studies by Luskey et al. (9) and Kudzma et al. (10) confirmed that VLDL synthesis was induced by estrogen. Bergink and co-workers (5) studied the kinetics of induction of the lipovitellin-phosvitin phosphoprotein complex in the rooster liver by estradiol. After a single injection of estradiol (25 mg/kg), the rate of synthesis of the complex was first shown to be elevated 4 h later, and the complex was detectable in blood 10 h after hormone treatment. Similarly, in studying the primary induction of another hepatic protein, phosvitin, by estrogen in the chicken, Jost et al. (28) found a lag phase of about 12 h before there was any accumulation of the phosphoprotein in the plasma. In addition to having a slower initial rate of induction compared to that of VLDL, the phosphoproteins appeared to stay in the circulation for a much longer time, 6–7 days according to the report of Bergink et al. (5). It appeared that

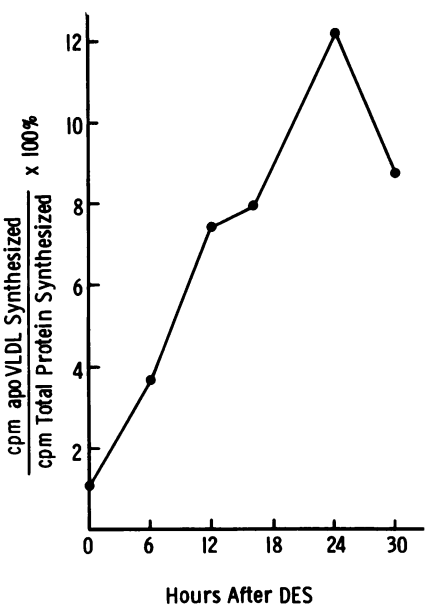

FIGURE 10 Effect of estrogen on mRNA activity of cockerel livers. Total protein vs. fraction B synthetic activity. Data from Fig. 9 were expressed as proportion of fraction B synthesized over total protein synthesized.

the estrogenic induction of VLDL is of much shorter duration (72 h) than that of phosvitin-lipovitellin. Furthermore, the VLDL accumulation can be totally accounted for by an increased rate of synthesis.

Bergink et al. (29) first presented evidence for the existence and possible induction of the yolk protein vitellogenin mRNA by estrogen. Jost et al. (6) also reported the accumulation of β_L-lipovitellin mRNA in the liver of immature chicks treated with estrogen. In neither case were the details of the induction kinetics studied. Furthermore, characterization of the in vitro translation product was not presented. In our study, nonspecific co-precipitation of counts in the antigen-antibody reaction was unlikely since rat testis mRNA, which resulted in similar TCA-precipitable counts in the wheat germ system, gave negligible counts on immunoprecipitation with fraction B antiserum. Moreover, the product of the in vitro translation was found to co-migrate with fraction B on SDS gel electrophoresis.

Reports from a number of laboratories have shown that steroid hormone induction of specific protein synthesis is associated with changes in the level of the corresponding mRNA. This is shown to be the case with the estrogen induction of ovalbumin (30–32), conalbumin, ovomucoid, and lysozyme (33, 34) and progesterone induction of avidin (30, 35, 36) in the chick oviduct, glucocorticoid induction of tryptophan oxygenase in the rat liver (37), androgen induction of α_{2u} globulin in the rat liver (38), and fructose diphosphate aldolase in the rat ventral prostrate (39). Chan et al. (30) found that estrogen-induced ovalbumin synthesis could be accounted for by an accumulation of translatable ovalbumin mRNA. An excellent correlation existed between extractable ovalbumin mRNA activity as directly quantitated by an in vitro translation system and intracellular mRNA activity as reflected by the rate of incorporation of [^{3}H]lysine into ovalbumin in oviduct slices (30). Probably the same type of correlation exisits in the case of apoVLDL mRNA accumulation and VLDL synthesis. In the latter instance, however, we were studying the accumulation of the mRNA for only one major VLDL protein (fraction B). Unfortunately, this protein accounted for only 40–50% of all the apoproteins in VLDL. From our data, we cannot draw any conclusions as to the other component(s). Furthermore, while it is possible that apoVLDL mRNA synthesis is an initial estrogen response, it is just as likely that the accumulation of the specific mRNAs is secondary to the effect of the hormone on lipid metabolism in the liver. Only detailed kinetic studies on the effects of estrogen on all the individual apoproteins and their mRNAs and on hepatic lipid synthesis and degradation will allow one to formulate a more definitive hypothesis as to which is the primary event in the estrogenic induction of VLDL synthesis in the cockerel liver.

In conclusion, we have presented evidence that estrogen induces VLDL synthesis in the cockerel liver. At least part of the increased rate of synthesis of VLDL observed is accounted for by an accumulation of a major apoVLDL mRNA. This observation provides a valuable tool for studying both the mechanism of action of estrogen in the chicken liver as well as the regulation of lipoprotein synthesis at a molecular level.

Note added in proof: The complete amino acid sequence of fraction B has recently been completed. (Jackson, R. L., H-Y. Lin, L. Chan, and A. R. Means. Manuscript in preparation.) Each monomer was found to consist of 82 amino acid residues linked by a single disulfide bond at residue 76, with a calculated mol wt of 9,444. The apoprotein behaves abnormally on SDS acrylamide gels accounting for the much lower apparent mol wt of ~6,000.

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REFERENCES

1. Nelson, G. J. 1972. Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism. John Wiley and Sons, Inc., New York. 980.
2. Wynn, V., J. W. H. Doar, G. L. Mills, and T. Stokes. 1969. Fasting serum triglyceride, cholesterol, and lipoprotein levels during oral contraceptive therapy. *Lancet.* **2**: 756–760.
3. Molitch, M. E., P. Oill, and W. D. Odell. 1974. Massive hyperlipemia during estrogen therapy. *J. Amer. Med. Assoc.* **227**: 522–525.
4. Wittliff, J. L., and F. T. Kenney. 1972. Regulation of yolk protein synthesis in amphibian liver. I. Induction of lipovitellin synthesis by estrogen. *Biochim. Biophys. Acta.* **269**: 485–492.
5. Bergink, E. W., H. J. Kloosterboer, M. Gruber, and G. Ab. 1973. Estrogen-induced phosphoprotein synthesis in roosters. Kinetics of induction. *Biochim. Biophys. Acta.* **294**: 497–506.
6. Jost, J.-P., G. Pehling, and O. G. Baca. 1975. Rate of synthesis of β_L-lipovitellin in the liver of immature chicks treated with 17β estradiol. *Biochem. Biophys. Res. Commun.* **62**: 957–965.
7. Bos, E. S., R. J. Vonk, M. Gruber, and G. Ab. 1972. Lipovitellin synthesizing polysomes: specific and quantitative isolation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **24**: 197–200.
8. Hillyard, L. A., C. Entenman, and I. L. Chaikoff. 1956. Concentration and composition of serum lipoproteins of cholesterol-fed and stilbestrol-injected birds. *J. Biol. Chem.* **223**: 359–368.
9. Luskey, K. L., M. S. Brown, and J. L. Goldstein. 1974. Stimulation of the synthesis of very low density lipo-

proteins in rooster liver by estradiol. *J. Biol. Chem.* **249**: 5939–5947.
10. Kudzma, D. J., P. M. Hegstad, and R. E. Stoll. 1973. The chick as a laboratory model for the study of estrogen-induced hyperlipidemia. *Metab. Clin. Exp.* **22**: 423–434.
11. Kekki, M., and E. A. Nikkilä. 1971. Plasma triglyceride turnover during use of oral contraceptives. *Metab. Clin. Exp.* **20**: 878–889.
12. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345–1353.
13. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**: 466–468.
14. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244**: 5687–5694.
15. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1970. Further characterization of apolipoproteins from the human plasma very low density lipoproteins. *J. Biol. Chem.* **245**: 6588–5694.
16. Gotto, A. M., W. V. Brown, R. I. Levy, M. E. Birnbaumer, and D. S. Fredrickson. 1972. Evidence for the identity of the major apoprotein in low density and very low density lipoproteins in normal subjects and patients with familial hyperlipoproteinemia. *J. Clin. Invest.* **51**: 1486–1494.
17. Jackson, R. L., and A. M. Gotto. 1972. A study of the cystine-containing apolipoprotein of human plasma high density lipoproteins: characterization of cyanogen bromide and tryptic fragments. *Biochim. Biophys. Acta.* **285**: 36–47.
18. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406–4412.
19. Liu, T.-Y., and Y. H. Chang. 1971. Hydrolysis of proteins with p-toluenesulfonic acid. Determination of tryptophan. *J. Biol. Chem.* **246**: 2842–2848.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagents. *J. Biol. Chem.* **193**: 265–275.
21. Manual of Laboratory Methods. 1972. Lipid Research Clinics Program. University of North Carolina, Chapel Hill, N. C.
22. Rosenfeld, G. C., J. P. Comstock, A. R. Means, and B. W. O'Malley. 1972. A rapid method for the isolation and partial purification of specific eucaryotic messenger RNAs. *Biochem. Biophys. Res. Commun.* **47**: 387–392.
23. Roberts, B. E., and B. M. Paterson. 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 2330–2334.
24. Means, A. R., S. Woo, S. E. Harris, and B. W. O'Malley. 1975. Estrogen induction of ovalbumin mRNA: evidence for transcription control. *Mol. Cell. Biochem.* **7**: 33–42.
25. Tomkins, G. M., T. D. Gelehrter, D. Granner, D. Martin, Jr., H. H. Samuels, and E. B. Thompson. 1969. Control of specific gene expression in higher organisms. Expression of mammalian genes may be controlled by repressors acting on the translation of messenger RNA. *Science (Wash. D. C.)*. **166**: 1474–1480.
26. Schjeide, O. A., and M. R. Urist. 1956. Proteins and calcium in serums of estrogen-treated roosters. *Science (Wash. D. C.)*. **124**: 1242–1244.
27. Hillyard, L. A., H. M. White, and S. A. Pangburn. 1972. Characterization of apolipoproteins in chicken serum and egg yolk. *Biochemistry.* **11**: 511–518.
28. Jost, J.-P., R. Keller, and C. Dierks-Ventling. 1973. Deoxyribonucleic acid and ribonucleic acid synthesis during phosvitin induction by 17β-estradiol in immature chicks. *J. Biol. Chem.* **248**: 5262–5266.
29. Bergink, E. W., R. A. Wallace, J. A. Van de Berg, E. S. Bos, M. Gruber, and G. Ab. 1974. Estrogen-induced synthesis of yolk proteins in roosters. *Am. Zool.* **14**: 1177–1193.
30. Chan, L., A. R. Means, and B. W. O'Malley. 1973. Rates of induction of specific translatable messenger RNAs for ovalbumin and avidin by steroid hormones. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1870–1874.
31. Rosenfeld, G. C., J. P. Comstock, A. R. Means, and B. W. O'Malley. 1972. Estrogen-induced synthesis of ovalbumin mRNA and its translation in a cell-free system. *Biochem. Biophys. Res. Commun.* **46**: 1695–1703.
32. Palmiter, R. D. 1973. Rate of ovalbumin messenger ribonucleic acid synthesis in the oviduct of estrogen-primed chicks. *J. Biol. Chem.* **248**: 8260–8270.
33. Palmiter, R. D., and L. T. Smith. 1973. Purification and translation of ovalbumin, conalbumin, ovomucoid and lysozyme messenger RNA. *Mol. Biol. Rep.* **1**: 129–134.
34. Palmiter, R. D., and L. T. Smith. 1973. Synergistic effects of oestrogen and progesterone on ovomucoid and conalbumin mRNA synthesis in chick oviduct. *Nat. New Biol.* **246**: 74–76.
35. O'Malley, B. W., G. C. Rosenfeld, J. P. Comstock, and A. R. Means. 1972. Steroid hormone induction of a specific translatable messenger RNA. *Nat. New Biol.* **240**: 45–48.
36. O'Malley, B. W., L. Chan, S. E. Harris, J. P. Comstock, J. M. Rosen, and A. R. Means. 1974. Molecular mechanisms of steroid-mediated alterations in target cell growth and differentiation: induction of specific messenger RNA's. *In* Control of Proliferaiton in Animal Cells. B. Clarkson, and R. Baserga, editors. Cold Spring Harbor Laboratories, New York. 85–96.
37. Schultz, G., M. Beato, and P. Feigelson. 1973. Messenger RNA for hepatic tryptophan oxygenase: its partial purification, its translation in a heterologous cell-free system, and its control by glucocorticoid hormones. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1218–1221.
38. Sippel, A. E., P. Feigelson, and A. K. Roy. 1975. Hormonal regulation of the hepatic messenger RNA levels for α_{2u}-globulin. *Biochemistry.* **14**: 825–829.
39. Mainwaring, W. I. P., F. R. Mangan, R. A. Irving, and D. A. Jones. 1974. Specific changes in the messenger ribonucleic acid content of the rat ventral prostate gland after androgenic stimulation. Evidence for the synthesis of aldolase messenger ribonucleic acid. *Biochem. J.* **144**: 413–426.