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# Apolipoprotein B of Avian Very Low Density Lipoprotein: Characteristics of Its Regulation in Nonstimulated and Estrogen-Stimulated Rooster†

Françoise Capony and David L. Williams*

ABSTRACT: The high molecular weight apolipoproteins of very low density lipoprotein (VLDL) have been examined in VLDL fractions from nonstimulated and estrogen-stimulated roosters. Apolipoprotein B (apo VLDL-B) as previously described [Williams, D. L. (1979) *Biochemistry 18*, 1056] was detected in nonstimulated as well as estrogen-stimulated animals. Comparison of apo VLDL-B species from nonstimulated and estrogen-stimulated animals showed no differences, suggesting that the same, or a very similar, apolipoprotein gene is expressed in the presence and absence of estrogens. Plasma apo VLDL-B was observed to accumulate 500-fold in response to a single treatment with diethylstilbestrol. A specific and quantitative assay has been developed to measure hepatic apo VLDL-B synthesis. This assay has been used to measure apo VLDL-B synthesis in liver slices from nonstimulated and estrogen-stimulated roosters as well as animals challenged with the antiestrogenic drug, tamoxifen. The results of these studies show that basal apo VLDL-B synthesis is 2–2.5% of liver protein synthesis in nonstimulated roosters. This level of synthesis is not effected by tamoxifen, indicating that basal apo VLDL-B synthesis is not due to low levels of endogenous estrogens. In response to estrogen treatment, apo VLDL-B synthesis increased sharply after a lag period of 1.5 h, reached a maximum at 15–24 h, and returned to the control level. At the response maximum apo VLDL-B synthesis was 12–15% of total liver protein synthesis. This response was completely blocked by tamoxifen. Calculation of the absolute increase in apo VLDL-B synthesis following estrogen treatment showed that increased hepatic synthesis alone cannot account for the 500-fold accumulation of apo VLDL-B in rooster plasma.

The association between hyperlipoproteinemic states and the development of cardiovascular disease has focused considerable attention on the structure and metabolic regulation of VLDL[1] (Goldstein & Brown, 1977; Schaefer et al., 1977; Osborne & Brewer, 1977). The importance of estrogenic hormones in human VLDL metabolism is evidenced by studies which have shown plasma triglycerides and VLDL to increase during therapy with estrogen-containing contraceptives (Wynn et al., 1969; Wallace et al., 1979). The increased incidence of cardiovascular complications associated with estrogen therapy (Kaplan, 1978; Shapiro et al., 1979) clearly emphasizes the need for a detailed understanding of VLDL metabolism and the alterations which occur in response to estrogens. The chicken has frequently been used for studies of VLDL since massive increases in plasma VLDL occur in response to exogenous estrogens in the rooster or with the onset of egg laying in the hen (Schjeide, 1954; Hillyard et al., 1956). A variety

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[1] Abbreviations used: VLDL, very low density lipoprotein; apo VLDL-B, apolipoprotein B of VLDL; apo VLDL-II, apolipoprotein II of VLDL; DES, *trans*-α,α′-diethyl-4,4′-stilbenediol; tamoxifen, *trans*-1-(*p*-dimethylaminoethoxyphenyl)-1,2-*trans*-diphenylbut-1-ene.

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of atherosclerotic lesions develop during this estrogen-dependent hyperlipoproteinemia (Chaikoff et al., 1948; Katz & Pick, 1961).

A major effect of estrogen is to increase the hepatic synthesis of VLDL lipids and apolipoproteins (Kudzma et al., 1973; Luskey et al., 1974). With respect to VLDL apolipoproteins, Chan and co-workers (1976) have used antibody against bulk VLDL to measure hepatic apolipoprotein biosynthesis and antibody against a specific apolipoprotein, apo VLDL-II, to measure cell-free translation products directed by RNA from estrogen-stimulated livers. Their work suggests that increased apolipoprotein biosynthesis reflects an accumulation of apolipoprotein mRNAs which are increased via mechanisms similar to those applicable to steroid action in other systems (Gorski & Gannon, 1976; Yamamoto & Alberts, 1976).

Major unresolved questions in this area concern the biosynthesis and metabolic regulation of individual apolipoproteins. Studies of this nature are clearly required in order to understand the hormonal regulation of VLDL metabolism as well as the participation of specific apolipoproteins in the biosynthesis, structure, and function of VLDL particles. Accordingly, this paper reports an analysis of apolipoprotein B in the nonstimulated and estrogen-stimulated rooster. Recent work from this laboratory (Williams, 1979) showed apolipoprotein B to be the only detectable high-molecular-weight apolipoprotein in VLDL fractions isolated after chronic stimulation with DES. Apolipoprotein B exhibited an apparent molecular weight of 350000 upon $NaDodSO_4$–polyacrylamide gel electrophoresis and represented 55% of total VLDL protein. The present study concerns three specific aspects of apolipoprotein B regulation: (1) whether apolipoprotein B is present in the VLDL fraction in the absence of estrogen treatment, (2) whether increased hepatic synthesis quantitatively accounts for plasma apolipoprotein B accumulation following estrogen treatment, and (3) whether hepatic apolipoprotein B synthesis in nonstimulated roosters is dependent upon endogenous estrogens.

## Experimental Procedures

*Analysis of Apo VLDL-B.* VLDL was isolated from plasma by ultracentrifugal floatation and stored as previously described (Williams, 1979). Plasma [$^3$H]VLDL was prepared from animals that received an intraperitoneal injection of 1 mCi of L-[4,5-$^3$H]leucine (60 Ci/mmol, New England Nuclear) 2 h prior to sacrifice. High-molecular-weight apolipoproteins were analyzed by polyacrylamide gel electrophoresis in $NaDodSO_4$ with electrophoretic system A (Williams, 1979). Apo VLDL-B in VLDL fractions from control and DES-treated roosters was quantitated by chromatography on Sepharose 6B columns (0.9 × 20 cm) in $NaDodSO_4$ (Williams, 1979). Sample loads of 1 mg of VLDL protein/0.4 mL were run, and recovery of total protein was greater than 90%. Chromatographic analysis of [$^3$H]VLDL was performed in the same fashion. Protein was measured (Lowry et al., 1951) with bovine serum albumin as standard.

Apo VLDL-B preparations from control and DES-treated animals were compared by the method of limited proteolysis mapping (Cleveland, 1977). Samples of apo VLDL-B isolated by chromatography in $NaDodSO_4$ were delipidated (Williams, 1979), adjusted to 1 mg/mL in the sample buffer of electrophoretic system A, and digested for 15 min–2 h with 0.1 mg/mL V8 protease at 37 °C. The peptides generated by proteolysis were analyzed by electrophoresis in 10% polyacrylamide gels (30:0.8 w/w acrylamide–bisacrylamide) containing $NaDodSO_4$ with the buffer system described by Laemmli (1970). The VLDL fractions from control and DES-treated roosters were compared by double-diffusion analysis against anti-apo VLDL-B as described (Williams, 1979). Rabbit antiserum against apo VLDL-B isolated from a DES-treated animal was prepared and characterized as described (Williams, 1979). This antiserum was free of detectable reactivity toward apo VLDL-II as judged by double-diffusion analysis, direct precipitin analysis, or indirect precipitin analysis employing goat anti-rabbit γ-globulin as the second antibody. The latter two analyses were carried out with [$^3$H]apo VLDL-II chromatographically isolated from [$^3$H]apo VLDL-B as described above.

*Measurement of Apo VLDL-B Synthesis.* White Leghorn roosters (SPAFAS, Norwich, CT) (0.4–0.6 kg) were injected intramuscularly with the indicated doses of DES (Sigma Chemical Co., St. Louis, MO) or tamoxifen citrate (ICI Americas, Inc., Wilmington, DE) in propylene glycol. Nonstimulated animals received an equal volume of vehicle. Subsequent to sacrifice, a 20-mg liver sample was removed, chopped into six pieces, and incubated for 1 h at 40 °C in 0.1 mL of Krebs–Ringer bicarbonate buffer (Umbreit et al., 1945) containing 50 μCi of L-[4,5-$^3$H]leucine (60 Ci/mmol, New England Nuclear) under an atmosphere of 95% $O_2$–5% $CO_2$. The tissue sample was then washed with 2 mL of iced Krebs–Ringer buffer, and subsequent procedures were carried out at 0–2 °C. The tissue sample was homogenized in 0.3 mL of 0.02 M sodium phosphate (pH 7), 0.15 M NaCl, 0.005 M ethylenediaminetetraacetic acid, 1% Triton X-100, and 100 μg/mL phenylmethanesulfonyl fluoride with six strokes of a motor-driven glass–Teflon tissue grinder. The homogenate was separated into supernatant and pellet fractions by centrifugation for 1 h at 226000*g*. The supernatant was either analyzed immediately or frozen in liquid $N_2$ and stored at −70 °C until analysis. The pellet fraction was dissolved in the sample buffer of electrophoresis system A for analysis as described below.

The immunoprecipitation assay was performed in 1.5-mL polypropylene microfuge tubes with 30 μL of tissue supernatant, 20 μL of anti-apo VLDL-B, and 1.2 μg of carrier VLDL. Anti-apo VLDL-B was treated with phenylmethanesulfonyl fluoride (100 μg/mL) for 30 min prior to use and adjusted to 1% Triton X-100. After incubation for 1 h at 0–2 °C, 1 mL of 0.02 M sodium phosphate (pH 7) and 0.15 M NaCl were added, and the immunoprecipitate was pelleted by centrifugation for 2 min at 10000*g*. The immunoprecipitate was washed twice with 1 mL of the same buffer and dissolved in 0.4 mL of Protosol (New England Nuclear), and the radioactivity was measured by liquid-scintillation spectrometry in a toluene-based fluid. Control precipitations were carried out with 20 μL of preimmune serum or with an antibovine albumin–bovine albumin combination. In the latter case, amounts of antiserum and antigen were used such that the mass of the immunoprecipitate was the same as that produced with the VLDL–anti-apo VLDL-B combination. Each tissue supernatant was assayed in triplicate and the anti-apo VLDL-B values were corrected by subtracting the value obtained in control precipitations. For routine analysis apo VLDL-B synthesis is expressed as a relative rate of synthesis. In this fashion [$^3$H]leucine incorporation into apo VLDL-B is given as a percentage of isotope incorporation into total supernatant protein. The rationale for this mode of data presentation is given below. Data are reported as the mean ± SEM of the number of animals employed. A minimum of three animals was used for each determination.

In several experiments, the rate of apo VLDL-B synthesis as measured by immunoprecipitation was compared with

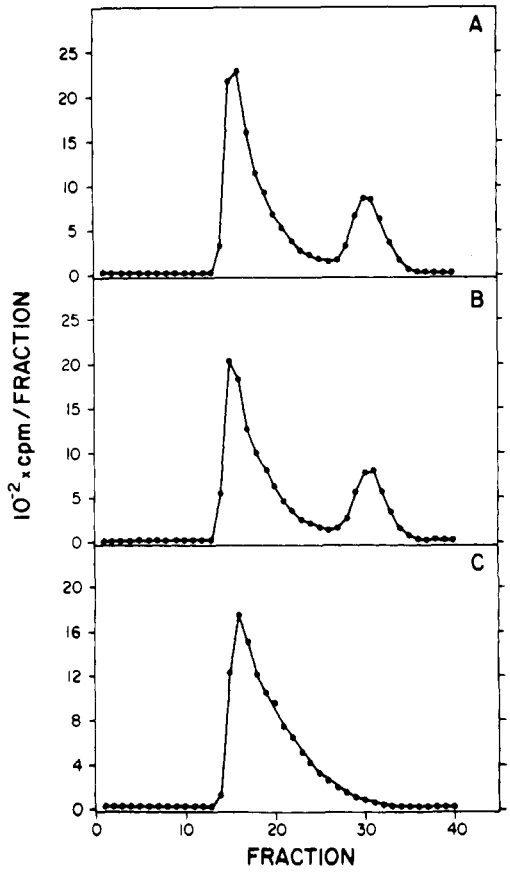


FIGURE 1: Elimination of apo VLDL-II coprecipitation. (A) [$^3$H]-VLDL (100 $\mu$g; 130 cpm/$\mu$g) was dissolved in 0.5 mL of 0.02 M sodium phosphate (pH 7), 1% $NaDodSO_4$, and 2% $\beta$-mercaptoethanol and chromatographed on a Sepharose 6B column equilibrated in 0.02 M sodium phosphate (pH 7) and 1% $NaDodSO_4$. Profile shows the chromatographic resolution of [$^3$H]apo VLDL-B (fraction 16) and [$^3$H]apo VLDL-II (fraction 30). (B) [$^3$H]VLDL (100 $\mu$g) was precipitated with 150 $\mu$L of anti-apo VLDL-B in 1 mL of 0.02 M sodium phosphate (pH 7) and 0.15 M NaCl. The immunoprecipitate was washed once with the same buffer, then dissolved, and chromatographed on Sepharose 6B as above. (C) [$^3$H]VLDL was immunoprecipitated as above except that the buffer contained 1% Triton X-100. The immunoprecipitate was dissolved and chromatographed on Sepharose 6B as above.

values obtained by electrophoresis. For this purpose parallel samples of supernatant were electrophoresed in system A (Williams, 1979), radioactive protein bands were visualized by fluorography (Bonner & Laskey, 1974), and the apo VLDL-B band was cut from the gel. The gel section was then combusted and radioactivity was recovered for scintillation counting as described (Williams et al., 1978a). This procedure was also used to assess the distribution of newly synthesized apo VLDL-B between the supernatant and pellet fractions prepared from the tissue homogenate. Electrophoretic analysis of the anti-apo VLDL-B immunoprecipitate was carried out in a similar fashion. Subsequent to electrophoresis, the gel lanes of interest were sliced into 3-mm sections and processed for the measurement of radioactivity as described above.

*Characterization of the Assay.* Immunoprecipitation of pulse-labeled proteins has been used to measure the synthesis of specific proteins in innumerable situations, and various criteria have been applied to validate the accuracy of such assays for particular proteins (see, for example, Palmiter, 1972). In the present study we encountered several unusual properties of the VLDL apolipoproteins relating to their solubility and association behavior, which necessitated a detailed characterization of the assay. These properties and the

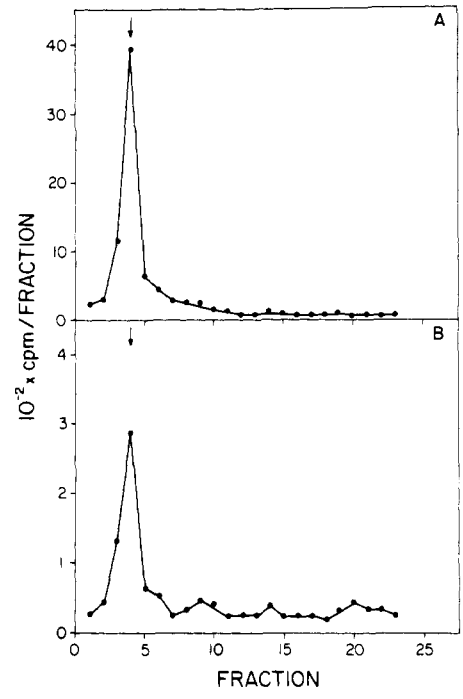


FIGURE 2: Electrophoretic profiles of anti-apo VLDL-B immunoprecipitates. Anti-apo VLDL-B immunoprecipitates formed with [$^3$H]leucine-labeled liver supernatants from DES-stimulated (A) and nonstimulated (B) roosters were run on a 5% polyacrylamide slab gel containing sodium dodecyl sulfate as described in Experimental Procedures. The arrow indicates the mobility of apo VLDL-B run in an adjacent gel lane.

assay characteristics are considered in four areas.

(*1*) *Assay Specificity.* Although antibody against apo VLDL-B showed no reactivity against isolated apo VLDL-II, we consistently observed precipitation of both apo VLDL-B and apo VLDL-II under certain conditions. The coprecipitation of apo VLDL-II is illustrated by the experiment with plasma [$^3$H]VLDL shown in Figure 1. Resolution of [$^3$H]-VLDL by chromatography on Sepharose 6B in $NaDodSO_4$ yields [$^3$H]apo VLDL-B (fraction 16) and [$^3$H]apo VLDL-II (fraction 30) (Figure 1A) as previously described (Williams, 1979). Immunoprecipitation of [$^3$H]VLDL with anti-apo VLDL-B yields both [$^3$H]apo VLDL-B and [$^3$H]apo VLDL-II in the precipitate when detergent is omitted from the reaction (Figure 1B) but only [$^3$H]apo VLDL-B when detergent is included (Figure 1C). These results suggest that apo VLDL-B and apo VLDL-II associate in a manner that is sensitive to detergent. Since we do not know the characteristics of this association, detergent was included in the immunoprecipitation assay to prevent it. For the assay conditions described above, 0.5–1% Triton X-100 was effective in preventing coprecipitation of newly synthesized apo VLDL-II. Immunoprecipitates formed with pulse-labeled proteins from DES-stimulated (Figure 2A) and nonstimulated (Figure 2B) livers showed only apo VLDL-B when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2) or chromatography on Sepharose 6B in $NaDodSO_4$ (data not shown).

(*2*) *Assay Quantitation.* Accurate measurement of newly synthesized apo VLDL-B required effective immunoprecipitation, complete extraction from the tissue, and an accurate assessment of nonspecific precipitation in the assay. Effective immunoprecipitation was found to require reactions at 0–2 °C in the presence of phenylmethanesulfonyl fluoride. In the absence of protease inhibitor or at elevated temperatures significant degradation of apo VLDL-B was observed. Reactions were designed such that the apo VLDL-B in the tissue

Table I: Comparison of Apo VLDL-B Synthesis by Electrophoresis and Immunoprecipitation[a]

| tissue | electrophoresis (% synthesis ± SEM) | immunoprecipitation (% synthesis ± SEM) | $N^b$ |
|---|---|---|---|
| DES-stimulated | 11.3 ± 0.8 | 10.4 ± 0.6 | 6 |
| nonstimulated | 2.6 ± 0.2 | 2.6 ± 0.1 | 4 |

[a] Tissue supernatants were prepared from DES-stimulated and nonstimulated liver slices which had been incubated for 1 h with [$^3$H]leucine as described under Experimental Procedures. Supernatant samples were analyzed by electrophoresis, and the radioactivity in the apo VLDL-B band was determined. Parallel supernatant samples were immunoprecipitated with anti-apo VLDL-B. A third set of supernatant samples was precipitated with trichloroacetic acid for the determination of [$^3$H]leucine incorporation into total supernatant protein. The values shown represent [$^3$H]-leucine incorporation into apo VLDL-B expressed as the percent of incorporation into total supernatant protein. [b] $N$ indicates the number of animals used in the measurements. Duplicate tissue incubations were carried out for each animal.

extract plus that added as carrier VLDL represented 25–50% of equivalence. Under these conditions [$^3$H]apo VLDL-B precipitation was maximal by 1 h, linearly related to the volume of extract assayed until equivalence was exceeded, and complete as judged by the quantitative recovery of apo VLDL-B added as carrier. The recovery of newly synthesized apo VLDL-B in the immunoprecipitate was also tested directly by comparison to the radioactivity in the apo VLDL-B band after electrophoresis of the tissue supernatant. As shown in Table I, apo VLDL-B synthesis by DES-stimulated and nonstimulated livers was the same with either method, showing directly the complete recovery of newly synthesized apo VLDL-B via immunoprecipitation.

Although rooster apo VLDL-B is quite soluble and stable in the presence of amphiphilic ligands (Williams, 1979), it is similar to human apolipoprotein B (Steele & Reynolds, 1979) in being almost totally insoluble in water or buffered solutions of common salts. As a result, liver extracts were prepared with homogenization buffer containing 1% Triton X-100. The efficiency with which newly synthesized apo VLDL-B was solubilized with this procedure was tested by electrophoretic analysis of pellet and supernatant fractions prepared from homogenates as described above. This analysis showed that apo VLDL-B was recovered in the supernatant with efficiencies of 96.2 ± 0.5% (SEM) and 92.1 ± 0.6% for DES-stimulated and nonstimulated livers, respectively. Corresponding values for extractions of total protein radioactivity were 89.9 ± 1.0% and 89.1 ± 0.7%.

Control assays to assess nonspecific immunoprecipitation yielded 0.1–0.2% of total supernatant protein radioactivity with extracts from DES-stimulated or nonstimulated livers. No difference was seen with either of the methods used to assess nonspecific precipitation. Comparison to values obtained with anti-apo VLDL-B (Table I) shows that nonspecific precipitation represents only 5–10% and 1–2% of uncorrected apo VLDL-B radioactivity with extracts from nonstimulated and DES-stimulated livers, respectively. The absence of significant nonspecific precipitation is also evident from the electrophoretic profiles shown in Figure 2.

*(3) Tissue Slice Incubations.* Under the conditions described, [$^3$H]leucine incorporation into total liver protein and apo VLDL-B proceeds linearly for 2 h. After 1 h, less than 5% of newly synthesized protein has been secreted while 15–25% has been secreted by 2 h. The utilization of [$^3$H]-leucine was examined with several culture media and serum concentrations as well as with the Krebs–Ringer buffer, which was used routinely. While minor differences in the absolute incorporation of precursor were noted, no significant differences in apo VLDL-B synthesis were observed when precursor incorporation into apo VLDL-B was expressed relative to incorporation into total protein.

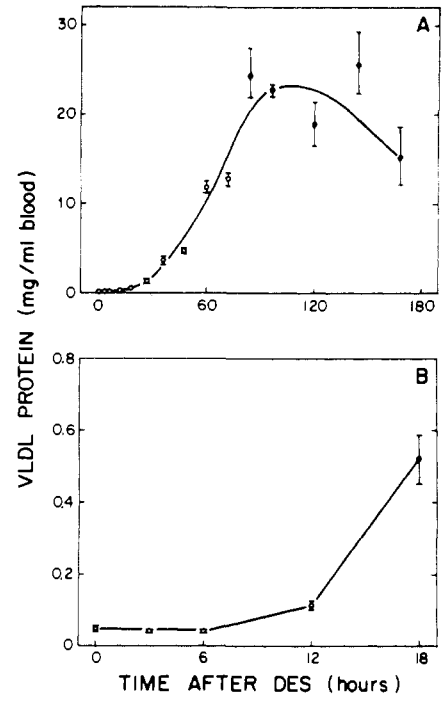


FIGURE 3: Accumulation of VLDL protein in rooster blood. (A) Roosters were treated with DES (25 mg/kg) at time 0, and VLDL was prepared from plasma at the indicated times. Data points show the mean (±SEM) VLDL protein/mL of blood for three animals at each time. (B) Data from panel A during the first 18 h after DES treatment.

*(4) Mode of Data Expression.* Apo VLDL-B synthesis is expressed with the relative-rate method since it involves only two straightforward measurements, is easily applied to large numbers of samples, and provides internal controls for the same variables which must be assessed directly when the absolute rate of synthesis is measured. The relative-rate method, for example, controls changes in precursor availability for apo VLDL-B synthesis by reference to precursor utilization for total protein synthesis. In this fashion, hormonal effects on precursor transport or variations in tissue-slice geometry which effect precursor uptake are internally controlled since such effects influence precursor incorporation into all proteins. Since total protein radioactivity and apo VLDL-B radioactivity are both recovered in the tissue extract with high efficiency (see above), the relative-rate method provides an accurate measurement of apo VLDL-B synthesis relative to total liver protein synthesis. Similarly, no major bias is introduced with leucine as precursor since the leucine content of apo VLDL-B (10.6 mol %, unpublished data) is fairly typical. Furthermore, the relative rate method is particularly advantageous for studies of apolipoprotein regulation because it yields a direct estimate of differential gene expression, thereby distinguishing specific hormonal effects from nonspecific effects which indiscriminately influence the synthesis of all proteins.

## Results

*VLDL Apolipoprotein B in Nonstimulated and DES-Stimulated Roosters.* As shown in Figure 3, total protein in the VLDL fraction increased from 50 μg/mL in nonstimulated animals to 25 mg/mL within 3–4 days following a single administration of DES (25 mg/kg). Electrophoretic analysis of VLDL fractions from nonstimulated animals showed a

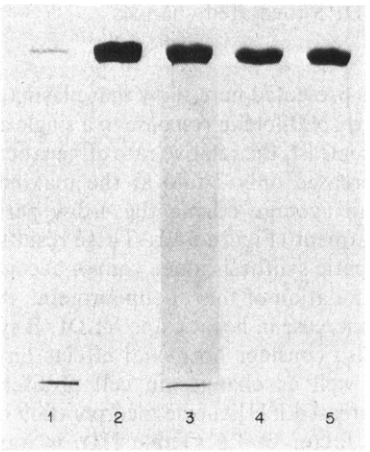


FIGURE 4: High-molecular-weight apolipoproteins of basal and DES-stimulated VLDL. VLDL samples (10–50 μg of protein) from the basal state (gel 1) and after DES stimulation for 12 (gel 2), 48 (gel 3), 63 (gel 4), and 72 h (gel 5) were run on 5% polyacrylamide slab gels containing sodium dodecyl sulfate as described (system A, Williams, 1979). In each case the observed high-molecular-weight band had the same mobility as apo VLDL-B run in an adjacent gel lane. The bottom of gels 1 and 3 shows the front at which apo VLDL-II runs in this gel system. For gels 2, 4, and 5, the front had run off the gels but bovine serum albumin ($M_r$ 65 000) was present at the bottom of marker lanes in these gels.

single high molecular weight apolipoprotein (Figure 4, lane 1) with the same mobility as the apo VLDL-B present in VLDL fractions at various times after DES treatment (Figure 4, lanes 2–5). The DES-stimulated increase in plasma apo VLDL-B was determined by chromatographic analysis of VLDL fractions from nonstimulated animals and at 3.5–4 days after DES treatment, at which point total VLDL protein had increased 500-fold (Figure 3). The apo VLDL-B concentration was 26 ± 2 (SEM) μg/mL of blood in nonstimulated animals and 12.5 ± 0.9 mg/mL of blood in DES-stimulated animals.

The extent of this increase in plasma apo VLDL-B prompted additional comparisons of the high molecular weight apolipoprotein from nonstimulated and DES-stimulated roosters. After digestion with *Staphylococcus aureus* V8 protease (Cleveland et al., 1977) and analysis of cleavage products by $NaDodSO_4$–10% polyacrylamide gel electrophoresis, identical cleavage patterns were observed for apo VLDL-B from nonstimulated roosters and apo VLDL-B from roosters 3 days after DES treatment. As many as 15–17 intermediate and core peptides were present in these digests (data not shown). VLDL fractions from nonstimulated and DES-stimulated animals were also compared by immunodiffusion analysis with anti-apo VLDL-B raised against apo VLDL-B from DES-stimulated animals. This analysis yielded a single line of identity between these samples with no evidence of spur formation (data not shown).

Table II: Lag Time for DES-Stimulated Apo VLDL-B Synthesis[a]

| time (h) | % synthesis ± SEM DES | control |
|---|---|---|
| 1 | 1.4 ± 0.1 | 1.7 ± 0.2 |
| 1.5 | 2.9 ± 0.4[b] | |
| 2 | 4.3 ± 0.3[b] | |
| 3 | 4.0 ± 0.3[b] | 1.5 ± 0.2 |

[a] Apo VLDL-B synthesis was assayed in liver slices from control or DES-treated animals (25 mg/kg) at the indicated times as described in Experimental Procedures except that the in vitro incubation period was 30 min. [b] These values differ from the controls at significance levels $P < 0.005$ (1.5 h) and $P < 0.001$ (2 h and 3 h) as judged by the Student's $t$ test.

*Regulation of Hepatic Apolipoprotein B Synthesis.* In the absence of exogenous hormone treatment, basal apo VLDL-B synthesis has been found to range between 1.5 and 2.5% of liver protein synthesis. Table II shows the results of an experiment carried out to determine the minimum period of DES stimulation required to increase apo VLDL-B synthesis. Apo VLDL-B synthesis was unchanged at 1 h but was clearly elevated at 1.5 h after DES treatment. Measurements of apo VLDL-B synthesis at longer intervals after DES treatment showed a maximum response by 24 h followed by a slow decline over the next 4 days to a rate about 4-fold above basal synthesis (Figure 5A). While large DES doses (25–100 mg/kg) yielded this prolonged elevation of apo VLDL-B synthesis, the response was of much shorter duration with lower doses. At DES doses of 1 mg/kg and 5–9 mg/kg, for example, apo VLDL-B synthesis had returned to the basal level by 48 and 72 h, respectively (data not shown). Throughout this dose range, however, apo VLDL-B synthesis was maximal at 15–24 h after DES treatment.

When apo VLDL-B synthesis was measured at 15 h, dose–response characteristics (Figure 5B) indicated a half-maximal increase at 0.5–1 mg/kg DES and a maximum

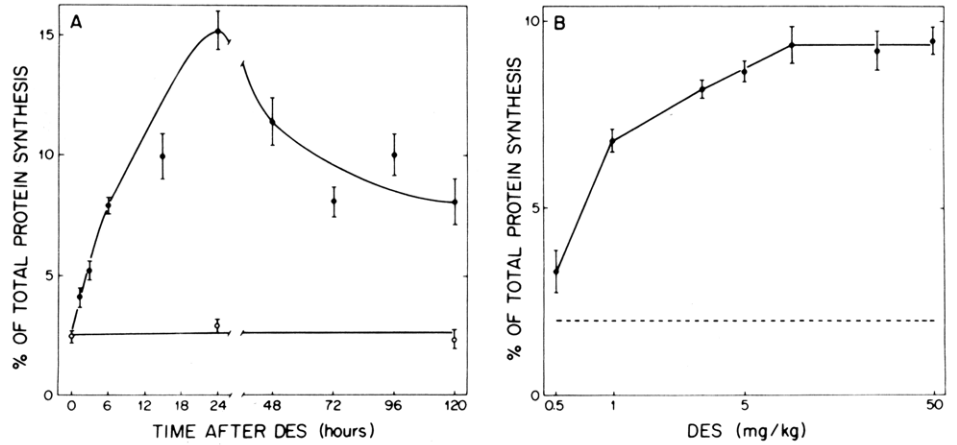


FIGURE 5: Time course and dose–response characteristics of DES-stimulated apo VLDL-B synthesis. (A) Roosters were injected with DES (25 mg/kg) (●) or vehicle (○) at time 0. At the indicated times, liver slices were incubated with [$^3$H]leucine, and the synthesis of apo VLDL-B was determined by immunoprecipitation. Data points show the mean (±SEM) for three animals per group. (B) Roosters were injected with the indicated doses of DES, and the synthesis of apo VLDL-B was determined at 15 h. Data points show the mean (±SEM) for three animals per group. The dashed line indicates the level of apo VLDL-B synthesis in nonstimulated animals. The abscissa is a logarithmic scale of DES dosage.

Table III: Influence of DES on [$^3$H]Leucine Incorporation into Total Liver Protein[a]

| DES dose (mg/kg) | incorporation ($10^{-3}$ × cpm/mg of protein ± SEM) |
|---|---|
| 0 | 1220 ± 80 |
| 1 | 1650 ± 200 |
| 25 | 1830 ± 280 |
| 50 | 1790 ± 70 |

[a] Roosters were injected with the indicated dose of DES, and the incorporation of [$^3$H]leucine into liver protein was determined at 15 h. The in vitro incubation period was 1 h.

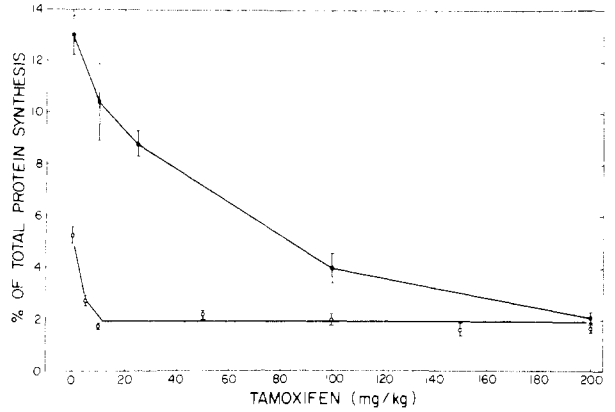


FIGURE 6: Tamoxifen inhibition of DES-stimulated apo VLDL-B synthesis. Tamoxifen citrate was administrated in the indicated doses simultaneously with DES at 25 mg/kg (●) or 1 mg/kg (○). Apo VLDL-B synthesis was determined at 48 h (●) or 15 h (○) after treatment. Data points show the mean (±SEM) for three animals per group.

synthesis of apo VLDL-B corresponding to about 10% of liver protein synthesis. We have observed essentially identical dose–response characteristics with equimolar doses of 17$\beta$-estradiol (data not shown). These data show that the hormone-stimulated increase in apo VLDL-B synthesis is both rapid and very substantial. The increase above basal apo VLDL-B synthesis at 15 h (Figure 5B) represents 8% of liver protein synthesis while the maximum increase at 24 h (Figure 5A) corresponds to about 13% of liver protein synthesis. In contrast to the marked stimulation of apo VLDL-B synthesis, DES yielded only a small increase in precursor incorporation into total liver protein. Table III shows data from a representative experiment in which a 1.5-fold increase was seen at 15 h after maximal doses of hormone.

In order to determine whether apo VLDL-B synthesis in nonstimulated animals is dependent on low levels of endogenous estrogens, DES-stimulated and nonstimulated animals were challenged with the antiestrogenic drug, tamoxifen. As shown in Figure 6, tamoxifen completely inhibited the increase in apo VLDL-B synthesis at DES doses of 1 mg/kg or 25 mg/kg, but tamoxifen did not decrease apo VLDL-B synthesis below the basal rate. When examined as a function of dose in nonstimulated animals ($\leq$200 mg/kg), tamoxifen showed no effect on the basal rate of apo VLDL-B synthesis measured at 15 h after tamoxifen treatment (data not shown). Similarly, when administrated at 50 mg/kg, tamoxifen did not alter the basal rate of synthesis over a 3-day period (data not shown). It should be noted that no changes in [$^3$H]leucine incorporation into total liver protein were observed as a result of tamoxifen administration to nonstimulated roosters. With DES-stimulated roosters, tamoxifen decreased [$^3$H]leucine incorporation by 30–40%, the extent of the inhibition being similar to the increase due to DES (Table III). The effects of tamoxifen, therefore, appear to be relatively specific and limited to antagonism of DES-mediated changes.

## Discussion

The results presented here show that plasma apo VLDL-B increased nearly 500-fold in response to a single administration of DES. In contrast, the relative rate of hepatic apo VLDL-B synthesis increased only 7-fold at the maximum and only 4.3-fold as an average during the 4-day period following hormone treatment (Figure 5A). These results suggest that increased hepatic synthesis alone cannot account for the extensive accumulation of this apolipoprotein. Assessment of the absolute increase in hepatic apo VLDL-B synthesis, however, must also consider hormonal effects on total protein synthesis as well as changes in cell proliferation. DES treatment increased [$^3$H]leucine incorporation into total liver protein by a factor of 1.5 (Table III), in agreement with numerous other studies (Dierks-Ventling & Jost, 1974; Luskey et al., 1974; Jost et al., 1975; Wachsmuth & Jost, 1976). When hormonal effects on the hepatic leucine pool are also considered (Dierks-Ventling & Jost, 1974), the effect on precursor incorporation indicates a 3–4-fold increase in liver protein synthesis. This stimulation is established quickly and is maintained for several days (Dierks-Ventling & Jost, 1974; Jost et al., 1975). An additional factor of 1.3 must be considered to account for increased liver DNA following estrogen stimulation (Schjeide & Lai, 1970; Jost et al., 1973). At the maximum, therefore, the 4.3-fold increase in the relative rate of apo VLDL-B synthesis may be adjusted to a 22-fold increase in the absolute rate of synthesis (4.3 × 4 × 1.3). While it is clear that estrogen markedly stimulates hepatic apo VLDL-B synthesis, it is equally clear from these considerations that factors other than hepatic synthesis are partially responsible for the 500-fold accumulation of this plasma apolipoprotein. Two possibilities may be suggested. First, we have recently noted that apo VLDL-B is also synthesized in the small intestine of the chicken (M. Blue and D. Williams, unpublished experiments). It is not presently known whether synthesis by the small intestine contributes significantly to the plasma accumulation of apo VLDL-B in estrogen-stimulated animals. Secondly, Kudzma et al. (1975) noted a 2–3-fold decrease in the fractional clearance of plasma VLDL-triglyceride in DES-stimulated roosters. Their results suggest that peripheral mechanisms for plasma VLDL clearance may become saturated at greatly elevated VLDL levels in estrogen-treated animals. Since the plasma VLDL levels produced in the present study are about 10-fold higher than those reported by Kudzma et al. (1975), saturation of clearance mechanisms may significantly contribute to the extensive accumulation of apo VLDL-B.

Analysis of VLDL fractions from nonstimulated animals showed the presence of an apo VLDL-B species which appears to be the same apolipoprotein that accumulates in response to estrogenic stimulation. As in the case after chronic DES stimulation (Williams, 1979), apo VLDL-B is the only detectable high molecular weight (>50 000) apolipoprotein in VLDL fractions from nonstimulated roosters as well as animals which received a single hormone treatment (Figure 4). The identity of apo VLDL-B species in nonstimulated and DES-stimulated VLDL is suggested by comparisons of electrophoretic mobility (Figures 2 and 4), immunological reactivity (Figure 2), and the sensitive method of limited proteolysis mapping (Cleveland et al., 1977). While these comparisons do not prove identity, they indicate that the nonstimulated and DES-stimulated apo VLDL-B species are very similar. This is a point of some interest since identity of these apo B species would indicate that the same apolipoprotein plays a role in

normal lipid metabolism in addition to its specialized role in the massive lipid mobilization required for egg yolk formation.

Vitellogenin is an egg yolk precursor protein of hepatic origin which has been extensively studied as a model for estrogen action in the liver (Bergink et al., 1974; Ryffel, 1978). Characteristics of apo VLDL-B regulation show interesting differences when compared to vitellogenin regulation. Of particular interest is estrogen-independent apo VLDL-B synthesis in nonstimulated animals. While tamoxifen acted as a pure antagonist for DES-stimulated apo VLDL-B synthesis (Figure 6), this antiestrogen had no influence on basal synthesis in nonstimulated animals, indicating that basal synthesis is not due to low levels of endogenous estrogens. This aspect of apo VLDL-B regulation is in sharp contrast to the complete estrogen dependence of vitellogenin synthesis and the accumulation of vitellogenin mRNA in rooster liver (Bergink et al., 1974; Burns et al., 1978; Jost et al., 1978; Williams et al., 1978b). Estrogen-stimulated apo VLDL-B synthesis also shows quantitative differences in comparison to vitellogenin induction. Whereas hepatic apo VLDL-B synthesis is markedly increased at 1.5 h after hormone treatment (Table II), vitellogenin synthesis is only detectable after a lag period of 4–5 h (Bergink et al., 1974; Jost et al., 1978). After the lag period, apo VLDL-B synthesis increases at 1.2–1.3%/h in terms of hepatic protein synthesis (Figure 5A, Table III) whereas vitellogenin synthesis increases at 0.15%/h during the first 24 h after estrogen treatment (Jost et al., 1978). After correction for the higher leucine content of apo VLDL-B (10.6 mol % compared to 8% for vitellogenin, Christmann et al., 1977), apo VLDL-B synthesis increases 7–8 times more rapidly than vitellogenin synthesis. The basis for these differences in the regulation of apo VLDL-B and vitellogenin is not known.

The relationship of the results presented here to previous studies of chicken VLDL is of interest. Chan and co-workers (1976, 1977) used antiserum against bulk VLDL to show increased apolipoprotein synthesis in response to DES and antagonism of this response by nafoxidine. Since the anti-VLDL antiserum used in those experiments (Chan et al., 1976) was not characterized with respect to its reactivity toward specific apolipoproteins, it is difficult to make specific comparisons with our results. It is unlikely, however, that apolipoprotein B was included in their measurements because of the procedures employed. The tissue extraction buffer, for instance, did not include detergent to permit extraction of the highly insoluble apo VLDL-B (Chan et al., 1976). Similarly, immunoprecipitations were carried out at elevated temperatures without protease inhibitors (Chan et al., 1976), conditions which lead to extensive degradation and poor recoveries of apo VLDL-B in our hands. The susceptibility of rooster apo VLDL-B to proteolytic degradation has been documented (Williams, 1979). As a result, it may be suggested that the increased apolipoprotein synthesis observed in their studies (Chan et al., 1976, 1977) was due to apo VLDL-II or other water-soluble apolipoproteins. In addition, these investigators used antibody specific to apo VLDL-II to show that RNA isolated from DES-stimulated livers directed the synthesis of apoVLDL-II in a cell-free translation system (Chan et al., 1976). These results clearly show that apo VLDL-II, as well as apo VLDL-B, is regulated by estrogens. It must be noted, however, that beyond this general conclusion little is known about the relationship between these two apolipoproteins. The extent to which the biosynthesis of these apolipoproteins is coordinated and the role of these apolipoproteins in VLDL structure and function have yet to be evaluated. Information of this nature is clearly required to elucidate the biosynthesis of VLDL particles as well as the mechanisms through which specific apolipoproteins are regulated.

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# Fluorescence Energy Transfer on Acetylcholinesterase: Spatial Relationship between Peripheral Site and Active Center†

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ABSTRACT: Spatial relationships between the peripheral site and active center on acetylcholinesterase have been examined with energy-transfer measurements by employing steady-state and time-resolved fluorescence spectroscopy. Active-center specific labeling with energy-transfer donors was accomplished with two distinct fluorescent phosphonates, the long-lived pyrenebutyl methylphosphonofluoridate and the shorter-lived (dansylamido)pentyl methylphosphonofluoridate. They react with the active-site serine of acetylcholinesterase to form fluorescent conjugates in a stoichiometry of one fluorophore per subunit. Propidium, a ligand selective for the peripheral site, serves as the energy-transfer acceptor and because it is fluorescent allows for alternative quantitation of the energy-transfer efficiency through sensitization of its emission following donor excitation. Steady-state quenching by propidium of the pyrene and dansyl conjugates occurs with 88 and 85% efficiencies, respectively. These efficiencies are in agreement with quenching values determined from analysis of the fluorescence lifetimes. The nanosecond decay rates of the conjugates measured in the absence of propidium do not display the exponential behavior observed for the free fluorophores but instead are characterized by two components. For the pyrene–enzyme conjugate two lifetimes ($\tau_1 = 80$; $\tau_2 = 160$ ns) are resolved in a ratio of amplitudes $a_1/a_2 = 3$, while for the dansyl enzyme the lifetimes ($\tau_1 = 6$; $\tau_2 = 20$ ns) are present as $a_1/a_2 = 0.33$. In the presence of propidium, the decay of the pyrene and dansyl conjugates is described by a function containing three exponential terms in which the two components having dominant amplitudes show considerably diminished lifetimes. Sensitization of acceptor fluorescence upon excitation of the pyrene donor is found to occur with only 35% efficiency compared with 85% donor quenching. The discrepancy in these values argues that propidium introduces quenching through nondipolar mechanisms as well as through excitation transfer. Maximum and minimum values for the orientation factor, $K^2$, were estimated from analysis of the individual donor and acceptor emission anisotropies, as well as from analysis of the transferred excitation between the pyrene–propidium pair. The intersite distance derived from examination of the pyrene–propidium pair is estimated to be $19 \leq R \leq 28$ Å, while that for the dansyl–propidium pair is $20 \leq R \leq 37$ Å. Association of bisquaternary ligands whose interquaternary distance extends 14 Å is mutually exclusive with binding of ligands at both the peripheral anionic site and the active center. Since an intersite distance exceeding 14 Å would preclude the bisquaternary ligands spanning between the sites, it is likely that a conformational change is associated with bisquaternary ligand binding. The altered conformation would either shorten the intersite distance or allosterically prevent the association of peripheral site ligands.

The 11S form of acetylcholinesterase (AchE)[1] isolated from *Torpedo* electric organs is a tetrameric enzyme composed of apparently equivalent, independent subunits of 80 000 mol wt (Taylor et al., 1974). Each subunit possesses an active center at which acetylcholine is hydrolyzed, as well as a peripheral anionic site which is spatially distinct from the active center (Changeux, 1966; Mooser & Sigman, 1974; Taylor & Lappi, 1975). Cationic ligands can alter catalysis by association at either the active center or the peripheral site, and formation of a ternary complex with active-center- and peripheral-site-selective ligands can be demonstrated. Cationic ligands that bind at the peripheral or active sites in a 1:1 stoichiometry with each subunit have been identified (Mooser & Sigman, 1974; Taylor & Lappi, 1975). Association of bisquaternary ammonium ligands of which decamethonium (I) is the prototype occurs with a 1:1 stoichiometry but is mutually exclusive with ligand association at both the active center and peripheral site (Taylor & Jacobs, 1974; Taylor & Lappi, 1975). Such an observation allows the inference that the maximum distance separating the active center and peripheral sites should be that conferred by the ten methylene carbon atoms separating the terminal cationic groups, i.e., 14 Å, a distance compatible with decamethonium spanning between the sites. In this paper steady-state and time-resolved fluorescence energy-transfer

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[1] Abbreviations used: AchE, acetylcholinesterase; PBMPF, pyrenebutyl methylphosphonofluoridate; $DC_5MPF$, [1-(dimethylamino)-naphthalene-5-sulfonamido]pentyl methylphosphonofluoridate or (dansylamido)pentyl methylphosphonofluoridate; PBMP-AchE, pyrenebutyl methylphosphonoacetylcholinesterase; $DC_5MP$-AchE, (dansylamido)-pentyl methylphosphonoacetylcholinesterase.

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