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Apoproteins of Avian Very Low Density Lipoprotein: Demonstration of a Single High Molecular Weight Apoprotein†

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ABSTRACT: The high molecular weight apoproteins of very low density lipoprotein (VLDL) were compared after preparation of VLDL from plasma and sera of diethylstilbestrol-treated roosters. When prepared from plasma with adequate control of endogenous proteolytic activity, VLDL contained a single high molecular weight apoprotein (apo-VLDL-B) as judged by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Serum VLDL contained multiple apoprotein species, the largest of which corresponded to apo-VLDL-B. Immunological analyses showed that the multiple apoproteins of serum VLDL were quantitatively and qualitatively indistinguishable from plasma apo-VLDL-B. These data indicate that apo-VLDL-B can be cleaved during VLDL isolation to produce an apparent heterogeneity of high molecular weight apoproteins. The molecular weight of plasma apo-VLDL-B was estimated to be 350 000. This protein was stable to reduction and S-carboxymethylation and showed no association with apo-VLDL-II [Chan, L., Jackson, R. L., O'Malley, B. W., & Means, A. R. (1976) *J. Clin. Invest. 58*, 368] through disulfide linkage. Apo-VLDL-B and apo-VLDL-II represented 54% and 46%, respectively, of the total VLDL protein recovered following gel filtration chromatography in sodium dodecyl sulfate. Protein recovery in the chromatographic analyses (92%) was sufficient to conclude that apo-VLDL-B and apo-VLDL-II are the major and possibly the only apoproteins of chicken VLDL. The molar ratio of the apo-VLDL-II monomer to apo-VLDL-B was estimated to be 32.

The association between hyperlipoproteinemic states and the development of atherosclerotic disease in man (Goldstein & Brown, 1977) has focused considerable attention on the structure and metabolic regulation of VLDL.[1] In this regard the chicken has frequently been employed for studies of VLDL. Blood levels of VLDL increase greatly with the onset of egg laying in the hen (Schjeide, 1954) or after estrogen administration to the rooster (Hillyard et al., 1956). Large quantities of chicken VLDL can be readily obtained for structural studies, and the hormonal regulation of VLDL synthesis can be studied in vivo (Luskey et al., 1974; Chan et al., 1976) or in cultured chick liver cells (Tarlow et al., 1977).

Studies of this nature require adequate knowledge of the individual protein moieties of VLDL. Until recently, however, little has been known about the number of apoproteins in VLDL or the characteristics of the individual protein species. There is general agreement that VLDL apoproteins fall into two very different molecular weight categories on the basis of gel-filtration chromatography in the presence of detergent (Hearn & Bensadoun, 1975; Chan et al., 1976; Chapman et al., 1977). A low molecular weight apoprotein, apo-VLDL-II, has recently been isolated, characterized, and sequenced (Chan et al., 1976; Jackson et al., 1977). This protein contains 82 amino acid residues, constitutes 40–50% of the total VLDL protein, and is probably the sole low molecular weight VLDL apoprotein. With regard to the high molecular weight apoproteins, however, there is little agreement as to the number of protein species in VLDL. In a recent study of hen VLDL (Chapman et al., 1977), $NaDodSO_4$ gel electrophoresis showed seven or more protein bands ranging in molecular weight from approximately 140 000 to over 300 000. Similar extents of

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[1] Abbreviations used: VLDL, very low density lipoprotein; apo-VLDL-B, apoprotein B of VLDL; apo-VLDL-II, apoprotein II of VLDL; DES, diethylstilbestrol; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; PSP, 0.02 M sodium phosphate (pH 7), 0.15 M NaCl, 100 μg/mL $PhCH_2SO_2F$; $NaDodSO_4$ sodium dodecyl sulfate.

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heterogeneity have been reported by others (Hillyard et al., 1972; Holdsworth et al., 1974; Hearn & Bensadoun, 1975). The marked heterogeneity of the high molecular weight VLDL apoproteins has complicated approaches to the structure of the VLDL particle and the metabolic regulation of individual apoprotein species.

In the present study the high molecular weight apoproteins have been characterized after preparation of VLDL from plasma and serum of estrogen-treated roosters. When prepared from plasma with adequate control of endogenous proteolytic activity, VLDL was found to contain a single high molecular weight apoprotein. This apoprotein has been characterized with respect to stability, molecular weight, its association with apo-VLDL-II, and its contribution to total VLDL protein. VLDL prepared from serum was found to contain multiple high molecular weight apoproteins, the largest of which corresponds to the single high molecular weight apoprotein of plasma VLDL. Immunological analyses were performed to determine whether the multiple apoproteins of serum VLDL were derived from the high molecular weight apoprotein through proteolytic cleavage during VLDL isolation.

Experimental Procedures

Preparation of VLDL. White leghorn roosters (SPAFAS, Norwich, CN) (1.1–1.6 kg) were injected intramuscularly with DES (50 mg/kg) in propylene glycol on day 0 and day 14. Three days after the second injection, animals were anesthetized with an intramuscular injection of sodium pentobarbital (100 mg/kg), the chest cavity was opened, and blood was drawn from the heart. For the preparation of serum, blood was clotted for 1 h at room temperature and centrifuged at 5000*g* for 10 min at 2 °C. For the preparation of plasma, blood was drawn into a small volume of sodium citrate containing a slurry of $PhCH_2SO_2F$ to yield final concentrations of 0.016 M sodium citrate and 200 μg/mL $PhCH_2SO_2F$. Cells were removed by centrifugation as above. VLDL was prepared by centrifugation at plasma or serum density for 40 h at 2 °C at 195000*g* in a Beckman SW-41 or SW-60 rotor. The floating pellicle of VLDL was removed, resuspended in PSP, and stored at 0–4 °C. Plasma [^{3}H]VLDL was prepared in the same fashion 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 VLDL Protein. For the preparation of antiserum against the high molecular weight protein of VLDL, VLDL was prepared from plasma as described above. The pellicle was suspended in PSP at 10 mg/mL of protein and a 7-mL sample was chromatographed on Sepharose 6B (2.6 × 30 cm) equilibrated in PSP at 0–4 °C. The leading 70% of the excluded peak was equilibrated with 0.05 M sodium citrate (pH 5.5), 100 μg/mL $PhCH_2SO_2F$, by dialysis, and chromatographed on diethylaminoethylcellulose (DE 52, Whatman) (2.6 × 20 cm) equilibrated in the same buffer at 0–4 °C. VLDL passes through this column while traces of contaminating vitellogenin are retained. The unbound VLDL fraction was dialyzed against four changes of 50 μg/mL $PhCH_2SO_2F$ (100 volumes each) in water at 0–4 °C, briefly warmed to room temperature, adjusted to 1% $NaDodSO_4$, and lyophilized. The sample was dissolved in 0.02 M sodium phosphate (pH 7), 3% $NaDodSO_4$, by gentle homogenization with a dounce homogenizer. A 7-mL sample (3 mg/mL) was chromatographed on Sepharose 6B (2.6 × 30 cm) equilibrated with 0.02 M sodium phosphate (pH 7), 1% $NaDodSO_4$, and 100 μg/mL $PhCH_2SO_2F$ at room temperature. After exposure to $NaDodSO_4$ VLDL chromatographs on this column as two well-resolved species. The high molecular weight component was concentrated by lyophilization and rerun on Sepharose 6B in $NaDodSO_4$ as above. The high molecular weight component from the second run was used for immunization as described below. This material was free of low molecular weight VLDL components as judged by electrophoresis in system B described below and by chromatography on analytical columns of Sepharose 6B (0.9 × 20 cm) in $NaDodSO_4$ under the conditions described above. By double-diffusion analysis, the high molecular weight VLDL component showed no reactivity toward antibodies raised against vitellogenin, α-lipovitellin, β-lipovitellin, or chicken serum albumin.

Immunological Procedures. Antiserum against the high molecular weight VLDL component was raised in New Zealand white rabbits by subcutaneous administration of antigen (0.9–1 mg) in Freund's complete adjuvant. Animals received booster injections at 3-week intervals and were bled 10 days after the third injection. Antiserum was adjusted to 0.02% NaN_3, cleared by centrifugation at 100000*g* for 1 h, and stored at 0–4 °C. Antiserum was treated with 100 μg/mL $PhCH_2SO_2F$ for 10 min at 30 °C and centrifuged at 10000*g* for 5 min before use. Direct precipitin analyses were performed in 50- or 100-μL volumes of PSP. After incubation for 2 h at 37 °C, the precipitate was removed by centrifugation for 5 min at 10000*g* and washed three times with 0.5 mL of PSP. The precipitate was dissolved in 0.1 N NaOH for the determination of protein or in Protosol (New England Nuclear) for the determination of radioactivity. Double-diffusion analyses were carried out in 1.3% agar gels in PSP for 1–2 days at room temperature.

Electrophoresis. System A. Protein samples were adjusted to 0.03 M tris(hydroxymethyl)aminomethane (pH 6.8), 3% $NaDodSO_4$, 0.01 M ethylenediaminetetraacetic acid, 2.5% β-mercaptoethanol, and 5% glycerol, boiled for 3 min, and run on 5% polyacrylamide slab gels containing 0.1% $NaDodSO_4$ with the discontinuous buffer system described by Laemmli (1970) for 20–30 h at 30 V. The stacking gel contained 4% acrylamide and the ratio of acrylamide to *N*,*N*′-methylenebisacrylamide was 30:0.8 in both running and stacking gels. Gels were fixed and stained for 1 h in 50% trichloroacetic acid containing 0.1% Coomassie blue and destained by diffusion in 7% acetic acid. For routine estimation of approximate molecular weights, the standard proteins (daltons) employed were myosin heavy chain (200000), β-galactosidase (116200), and chicken serum albumin (65000). Since the high molecular weight protein of plasma VLDL has a slower mobility than myosin, a series of molecular weight estimations were performed with standard proteins of higher molecular weight. For this purpose, phosphorylase A was cross-linked with dimethyl suberimidate as described by Davies & Stark (1970). Upon electrophoresis of the cross-linked phosphorylase A in system A, four bands were observed, the mobilities of which yielded a straight line when plotted against the logarithms of multiples of the molecular weight of phosphorylase A. Since this line was coincident with the standard curve for myosin, β-galactosidase, and serum albumin, the four phosphorylase A bands were assumed to represent the monomer, dimer, trimer, and tetramer of the phosphorylase A subunit with respective molecular weights of 97400, 194800, 292200, and 389600. For the measurement of radioactivity, the gel was dried, the lane of interest was cut into 6-mm sections, and the gel sections were combusted in a sample oxidizer and recovered for scintillation counting as described (Williams et al., 1978).

System B. Low molecular weight proteins were run on 20% polyacrylamide slab gels containing 1% $NaDodSO_4$ with the buffer system of Fairbanks et al. (1971). A 4% acrylamide

stacking gel was employed with the same buffer components adjusted to pH 6.5 with acetic acid. The *N,N'*-methylenebisacrylamide concentration was 0.125% for both the stacking and running gels. Protein samples were adjusted to 0.01 M tris(hydroxymethyl)aminomethane (pH 6.5 with acetic acid), 2% $NaDodSO_4$, 2% β-mercaptoethanol, 0.001 M ethylenediaminetetraacetic acid, and 4% glycerol and boiled for 3 min prior to electrophoresis. Pyronin Y was used as the tracking dye in separate slots since it was found to bind to the low molecular weight VLDL proteins and distort the bands. Gels were run for 16–18 h at 30 V. Subsequent to electrophoresis the gels were fixed with a modification of the glutaraldehyde procedure described by Keck et al. (1973). The gel was washed twice at room temperature for 15 min with 20 volumes of 0.02 M sodium phosphate (pH 7), incubated with 5% glutaraldehyde in the same buffer for 2 h, washed again for 15 min in buffer, and incubated with 0.2 mg/mL KBH_4 in water for 2 h. The gel was then stained and destained as described above. This procedure was used because the low molecular weight VLDL proteins are soluble in acid and slowly wash out of the gels during destaining. Fixation with glutaraldehyde prior to staining was found to markedly increase the staining intensity of these bands.

S-Carboxymethylation. [^{3}H]VLDL was prepared and separated into high and low molecular weight components by chromatography on Sepharose 6B in $NaDodSO_4$ as described above. Both fractions were precipitated with 80% acetone and sequentially extracted with ethanol, chloroform:ethanol (2:1), ethanol, ethanol:diethyl ether (1:1), and ether. The extracted residues were dissolved in 3% $NaDodSO_4$, reduced, and S-carboxymethylated as described (Means & Feeney, 1971). The samples were chromatographed on Sephadex G-25 in 0.005 M sodium phosphate (pH 7), 1% $NaDodSO_4$, and aliquots of the excluded peak were examined by electrophoresis in systems A and B as described above. When samples were run in system B, the gel lanes of interest were immediately dried after electrophoresis, and radioactivity was measured in gel sections as described above.

Miscellaneous. Buffers and glassware were sterilized by autoclaving, and centrifuge tubes were soaked in 1 N NaOH and rinsed with sterile water before use. $PhCH_2SO_2F$ was added to solutions immediately before use. Protein was measured (Lowry et al., 1951) with bovine serum albumin as standard. Radioactivity in aqueous samples was measured by scintillation spectrometry in Aquasol (New England Nuclear) or a toluene-based counting fluid containing 6% Protosol.

Results

Comparison of Plasma VLDL and Serum VLDL Apoproteins. Figure 1 shows the electrophoretic profiles in system A of VLDL proteins prepared from plasma and serum of the same animal. Plasma VLDL (lane 1) shows a single high molecular weight protein. By extrapolation from the mobilities of standard proteins run on this gel, the high molecular weight component of plasma VLDL has an approximate molecular weight of 350 000. The bottom of this gel corresponds to a molecular weight of approximately 50 000. In contrast to the single high molecular weight protein observed with plasma VLDL, serum VLDL (lane 2) shows a reduced amount of the band corresponding to the plasma VLDL protein, a second major protein of approximate molecular weight 290 000, and a number of other proteins ranging in molecular weight down to approximately 100 000. The only differences in the preparation of serum and plasma VLDL were the use of citrate to prevent coagulation and the presence of $PhCH_2SO_2F$ to inhibit proteolysis in the plasma sample.

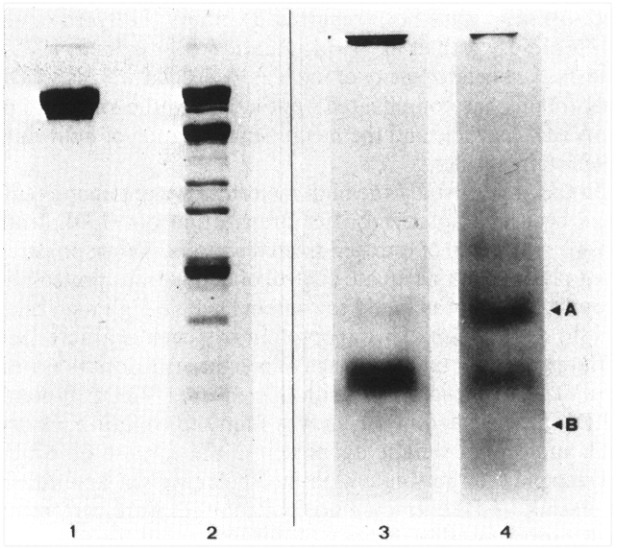

FIGURE 1: Electrophoretic comparison of the apoproteins of plasma VLDL and serum VLDL. Plasma VLDL and serum VLDL were prepared from blood drawn from the same animal. Samples containing 50 μg of plasma VLDL (lanes 1 and 4) or serum VLDL (lanes 2 and 3) protein were analyzed by electrophoresis in systems A (lanes 1 and 2) and B (lanes 3 and 4). The bottom of lanes 1 and 2 corresponds to a molecular weight of 50 000. Lanes 3 and 4 were fixed with the glutaraldehyde procedure and stained as described in Experimental Procedures. The arrows show the migration positions of ribonuclease (arrow A) and insulin (arrow B) for lanes 3 and 4.

Analysis of plasma VLDL and serum VLDL by electrophoresis in system B shows little or no difference in the low molecular weight components (Figure 1, lanes 3 and 4). Both serum VLDL and plasma VLDL show two low molecular weight bands, the larger of which runs with ribonuclease (arrow A) and the smaller of which runs between ribonuclease and insulin (arrow B). These bands appear to represent the monomer–dimer forms of apo-VLDL-II which has previously been shown to be one of the principal VLDL proteins (Chan et al., 1976; Jackson et al., 1977). These bands will be referred to as apo-VLDL-II. Additional evidence that these bands are apo-VLDL-II is given below. Note that in this gel system the high molecular weight protein of plasma VLDL and the multiple high molecular weight proteins of serum VLDL remain at the top of the running gel.

The electrophoretic comparisons of plasma VLDL and serum VLDL (Figure 1) indicate that serum VLDL contains a number of proteins not seen in plasma VLDL. The additional protein bands of serum VLDL are primarily confined to the molecular weight range greater than 100 000. Since a single high molecular weight protein was observed when VLDL was prepared from plasma in the presence of $PhCH_2SO_2F$, it seemed likely that the multiple high molecular weight proteins of serum VLDL were derived from the 350 000 mol wt component through proteolysis. In order to test this point, antiserum was raised against the high molecular weight protein of plasma VLDL which was purified as described in Experimental Procedures. As shown in Figure 2, this antiserum yields a single precipitin line when tested by double-diffusion analysis against its antigen but shows no reactivity toward the low molecular weight apo-VLDL-II. When tested against the high molecular weight protein of plasma VLDL and intact plasma VLDL, a single line of identity was also observed (data not shown). Quantitative precipitin analyses were performed with this antiserum and the plasma VLDL and serum VLDL preparations described in Figure 1. The data of Figure 3 show that plasma VLDL and serum VLDL have the same equivalence value with this antiserum and yield

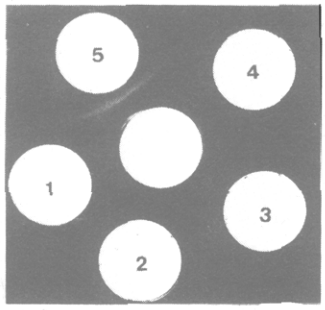

FIGURE 2: Double-diffusion analysis of high and low molecular weight plasma VLDL proteins. The high and low molecular weight plasma VLDL proteins as described in Figure 1 were separated by chromatography on Sepharose 6B in $NaDodSO_4$ as described in Experimental Procedures. The two fractions were concentrated by centrifugation at 310000*g* for 24 h at 20 °C in a Beckman SW-60 rotor and readjusted to 5 mg/mL with PSP to yield a final $NaDodSO_4$ concentration of 0.05%. Additional dilutions were made with PSP. Each well contained 4 μL of the following: (wells 1–4) low molecular weight apo-VLDL-II fraction at 2, 1, 0.5, and 0.25 mg/mL, respectively; (well 5) high molecular weight VLDL fraction at 1 mg/mL; (center well) antiserum against the high molecular weight VLDL protein.

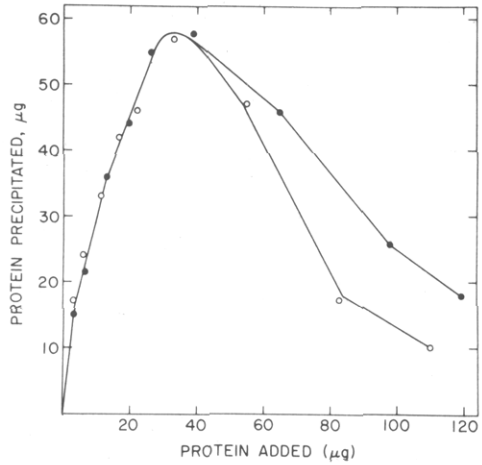

FIGURE 3: Precipitin analysis of plasma VLDL and serum VLDL. The plasma and serum VLDL samples described in Figure 1 were reacted with 30 μL of antiserum in 100-μL assays as described in Experimental Procedures. Data points are means of duplicate determinations for plasma VLDL (●—●) and serum VLDL (○—○).

the same amount of protein in the immunoprecipitate at equivalence. The immunological similarity of plasma VLDL and serum VLDL was also tested by comparing their behavior in the region of antigen excess. For this purpose the antiserum was incubated with an amount of plasma [^{3}H]VLDL slightly less than necessary to yield equivalence plus additional quantities of plasma [^{3}H]VLDL, unlabeled plasma VLDL, or unlabeled serum VLDL. The data of Figure 4 show that as the amount of additional protein was increased into the region of antigen excess, the precipitation of input plasma [^{3}H]VLDL decreased in a quantitatively similar fashion irrespective of whether the additional protein was serum VLDL, plasma VLDL, or plasma [^{3}H]VLDL.

The data of Figures 3 and 4 show that plasma VLDL and serum VLDL behave in a quantitatively indistinguishable fashion when tested with antiserum raised against the high molecular weight protein of plasma VLDL. In order to determine directly in a qualitative fashion that the multiple high molecular weight proteins of serum VLDL are precipitated by the antiserum, the immunoprecipitates formed with plasma VLDL and serum VLDL were compared by electrophoresis in system A. The gel patterns show that most of the multiple

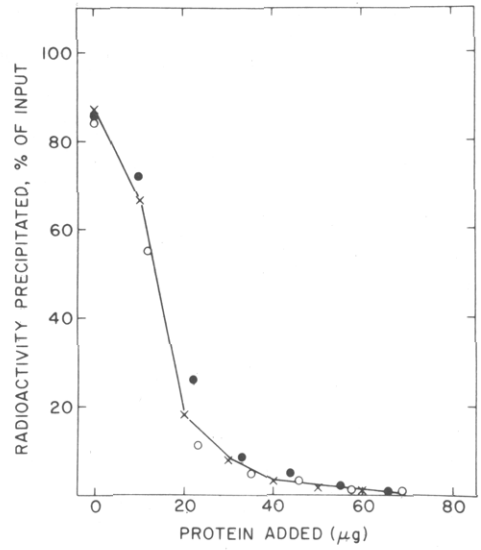

FIGURE 4: Behavior of plasma VLDL and serum VLDL in the region of antigen excess. Antiserum against the high molecular weight plasma VLDL protein was incubated with 10 μg of [^{3}H]VLDL (100 cpm/μg) and the indicated additional amounts of plasma [^{3}H]VLDL (X—X), ılabeled plasma VLDL (●—●), or unlabeled serum VLDL (○—○). ssays were carried out in duplicate with 15-μL antiserum in 50-μL lumes. The unlabeled plasma and serum VLDL samples are those scribed in Figure 1. The immunoprecipitates were dissolved in otosol for the measurement of radioactivity.

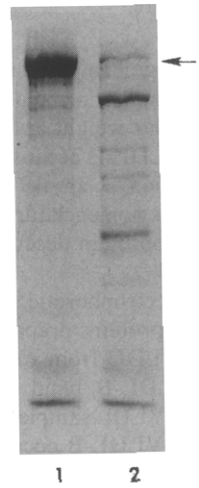

FIGURE 5: Electrophoretic analysis of plasma VLDL and serum VLDL immunoprecipitates. Antiserum (30 μL) against the high molecular weight plasma VLDL protein was reacted with 44 μg of plasma VLDL or serum VLDL in 100-μL assays as described in Experimental Procedures. The immunoprecipitates were dissolved in electrophoresis sample buffer and run in system A. (Lane 1) Plasma VLDL immunoprecipitate; (lane 2) serum VLDL immunoprecipitate. The bottom of the gel corresponds to a molecular weight of approximately 50 000. The two fastest running bands at the bottom of each lane are from the antiserum. The arrow indicates the mobility of apo-VLDL-B in a sample of VLDL which was not immunoprecipitated.

protein bands of serum VLDL (Figure 5, lane 2) are present in the immunoprecipitate. Note that some degradation of the high molecular weight plasma VLDL protein occurs during immunoprecipitation (Figure 5, lane 1), but most of the protein remains as a single high molecular weight species.

On the basis of these immunological analyses, the multiple high molecular weight proteins of serum VLDL appear to be quantitatively (Figure 3 and 4) and qualitatively (Figure 5) indistinguishable from the single high molecular weight protein of plasma VLDL. These data provide strong evidence that the multiple protein species observed in VLDL prepared from serum are derived from a single high molecular weight protein

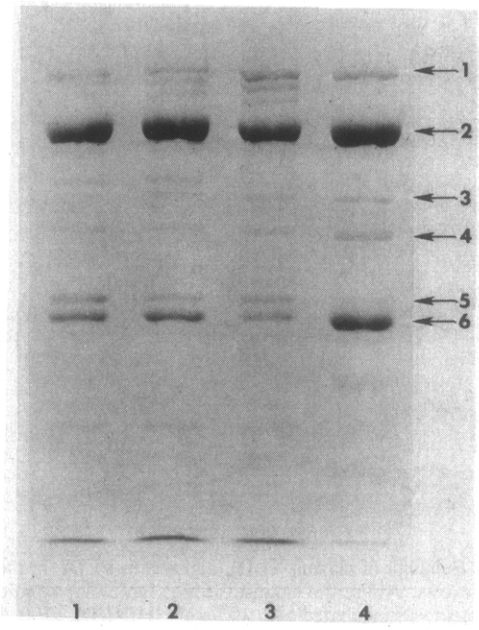

FIGURE 6: Electrophoretic comparison of serum VLDL. Serum VLDL was prepared from four roosters and analyzed by electrophoresis in system A. Each sample contained 50 μg of VLDL protein. Protein bands labeled 1–6 are described in the text. The bottom of this gel corresponds to a molecular weight of approximately 65000. The serum VLDL described in Figure 1 (lane 2) is from a different rooster than that of the samples shown here.

through proteolytic cleavage. In previous studies of chicken VLDL, the largest of the high molecular weight apoproteins or the entire high molecular weight apoprotein fraction has been referred to as apo-VLDL-B because of similarities with human apo-VLDL-B (Chan et al., 1976; Chapman et al., 1977). In keeping with this nomenclature, the high molecular weight plasma VLDL apoprotein described here will be referred to as apo-VLDL-B.

Figure 6 shows an electrophoretic comparison of high molecular weight VLDL proteins prepared from the sera of four roosters. Plasma VLDL from each of these animals showed a single apo-VLDL-B band as described above. Comparison of the serum VLDL samples (Figure 6) indicates that the cleavage of apo-VLDL-B occurs in a qualitatively reproducible fashion from animal to animal. The yields of the various bands clearly differ, but all of the major bands and most of the minor bands can be seen in each sample. Band 1 represents intact apo-VLDL-B, and bands 2–6 are the major cleavage products with approximate molecular weights of 290000 (2), 250000 (3), 210000 (4), 165000 (5), and 155000 (6). Band 2 is the primary product formed in each case and appears to persist even when only a small amount of intact apo-VLDL-B remains (lane 4). In a similar fashion band 6 was found to be greatly enhanced in samples with little intact apo-VLDL-B remaining. These features suggest that some of the apo-VLDL-B cleavage products are relatively resistant to further breakdown.

As in the case with the estrogen-treated rooster, plasma VLDL prepared from the laying hen showed a single high molecular weight protein with the same mobility as rooster apo-VLDL-B when examined by electrophoresis in system A (data not shown). Serum VLDL prepared from the laying hen showed multiple high molecular weight proteins with electrophoretic mobilities similar to those of the multiple proteins of serum VLDL prepared from estrogen-treated roosters. Plasma VLDL and serum VLDL have also been prepared from roosters and 3-week-old chicks which have not been treated with diethylstilbestrol. In these cases both plasma VLDL and serum VLDL showed predominantly a single high molecular weight protein with the same electrophoretic mobility as apo-VLDL-B from estrogen-treated roosters (data not shown). With several control animals, serum VLDL showed bands corresponding to bands 2 and 3 of serum VLDL prepared from hormone-treated roosters. In general, however, these bands were minor components of serum VLDL from control animals. Since the blood levels of VLDL in control and hormone-treated animals differ by as much as 100 fold, the absence of extensive apo-VLDL-B cleavage in control sera may indicate a VLDL concentration dependence for this process.

Characteristics of Rooster Apo-VLDL-B. When isolated from plasma in the presence of $PhCH_2SO_2F$, apo-VLDL-B appears to be quite stable when stored as intact VLDL. Plasma VLDL has been kept at 0–4 °C in the presence of $PhCH_2SO_2F$ for several months with no significant breakdown of apo-VLDL-B. Similarly, after separation of apo-VLDL-B from apo-VLDL-II by gel-filtration chromatography in $NaDodSO_4$, apo-VLDL-B has shown no significant breakdown during repeated chromatography. Electrophoresis in system A of isolated apo-VLDL-B, or intact VLDL after treatment with $NaDodSO_4$, yields a single high molecular weight apo-VLDL-B band as described above. Protein components of slower electrophoretic mobility have not been observed and only traces of protein stain have been seen at the origin or the running gel interface in system A unless the sample load exceeded 80–100 μg/slot. Similarly, after acetone precipitation, or the more extensive lipid extraction procedure described in Experimental Procedures, the electrophoretic mobility of apo-VLDL-B in system A was unchanged.

When examined by electrophoresis in system A, the mobility of apo-VLDL-B was the same with or without prior reduction with β-mercaptoethanol. Similarly, electrophoretic analysis in systems A and B of the apo-VLDL-B and apo-VLDL-II fractions prepared by chromatography on Sepharose 6B in $NaDodSO_4$ showed no significant cross-contamination of these proteins. These results suggest that apo-VLDL-B is not associated with itself or with apo-VLDL-II through disulfide bonds. Since reduced apo-VLDL-II is known to reoxidize to dimers during electrophoresis (Chan et al., 1976), however, the possibility that some apo-VLDL-II is associated with apo-VLDL-B through disulfide linkage was tested in more detail. For this purpose plasma [^{3}H]VLDL was prepared and separated into apo-VLDL-B and apo-VLDL-II by chromatography on Sepharose 6B in 1% $NaDodSO_4$. Figure 7 shows the resolution of plasma [^{3}H]VLDL into these two fractions. The high molecular weight apo-VLDL-B fraction was reduced, S-carboxymethylated, and examined by electrophoresis in systems A and B. Figure 8 shows the distribution of radioactivity in system A of the control and S-carboxymethylated [^{3}H]apo-VLDL-B. No significant change in the electrophoretic profile was seen in the S-carboxymethylated sample, and no significant radioactivity was detected in the region corresponding to the mobility of apo-VLDL-II in this system (fraction 12). The S-carboxymethylated [^{3}H]apo-VLDL-B was also run in system B, and the gel region corresponding to the mobilities of the apo-VLDL-II bands was sectioned and counted (Figure 8, insert). No significant radioactivity was observed in this gel region in the control or S-carboxymethylated sample. These data indicate that [^{3}H]apo-VLDL-B is stable to reduction and S-carboxymethylation and shows no significant association with apo-VLDL-II through disulfide linkage.

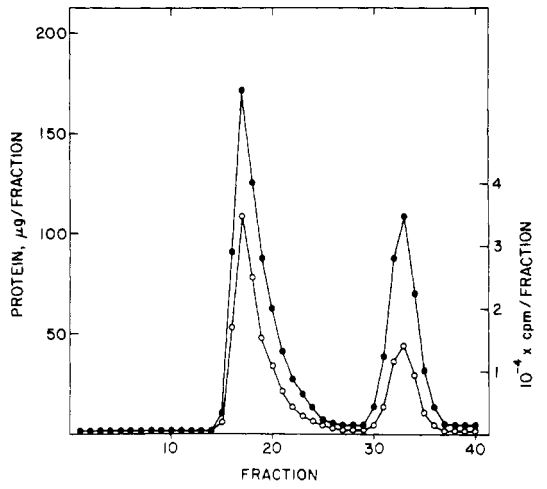

FIGURE 7: Chromatographic separation of apo-VLDL-B and apo-VLDL-II fractions. Plasma [^{3}H]VLDL (1.2 mg of protein) was adjusted to 0.02 M sodium phosphate (pH 7), 3% $NaDodSO_4$, and chromatographed on Sepharose 6B (1.5 × 20 cm) equilibrated with 0.02 M sodium phosphate (pH 7), 1% $NaDodSO_4$. Fractions were assayed for protein and radioactivity. The peak eluting at fraction 18 is apo-VLDL-B, while the peak at fraction 33 is apo-VLDL-II. Electrophoresis of these peaks in systems A and B showed no significant cross-contamination of apo-VLDL-B and apo-VLDL-II. Recoveries of protein (●—●) and radioactivity (○—○) were 88% and 87%, respectively.

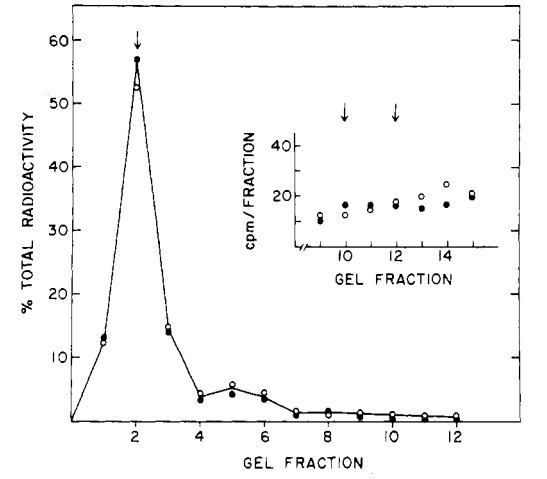

FIGURE 8: S-carboxymethylation of apo-VLDL-B. [^{3}H]Apo-VLDL-B was prepared from plasma [^{3}H]VLDL by chromatography on Sepharose 6B in $NaDodSO_4$ as described in Figure 7. [^{3}H]Apo-VLDL-B was delipidated and S-carboxymethylated as described in Experimental Procedures. Samples of the control (●—●, 5000 cpm) and S-carboxymethylated (○—○, 3500 cpm) protein were reduced and electrophoresed in system A. The arrow indicates the gel fraction corresponding to the mobility of undelipidated apo-VLDL-B from a sample of unlabeled VLDL run in an adjacent gel lane. Apo-VLDL-II was in fraction 12 and is not resolved into two bands in system A. (Insert) The same amounts of the control (●—●) and S-carboxymethylated (○—○) [^{3}H]apo-VLDL-B were run in system B. These gel lanes were not fixed, but were dried, sectioned, and combusted after electrophoresis. Unlabeled VLDL was run in an adjacent lane which was fixed and stained. The arrows mark the gel fractions corresponding to the two apo-VLDL-II bands in the stained lane.

When compared with the electrophoretic mobilities of myosin, β-galactosidase, and serum albumin in system A, the mobility of apo-VLDL-B yielded an apparent molecular weight of approximately 350000. Since the mobility of apo-VLDL-B falls well outside the range of these marker proteins, high molecular weight protein standards were constructed by cross-linking the phosphorylase A tetramer with dimethyl suberimidate by the procedure of Davies & Stark (1970).

Table I: Protein Composition of Rooster VLDL[a]

sample	$\bar{M}$ ± SD
total VLDL protein (mg/mL of blood)	23 ± 4
apo-VLDL-B protein (% of total)	54 ± 2
apo-VLDL-II protein (% of total)	46 ± 2
recovery (%)[b]	92 ± 5

[a] Plasma VLDL was prepared from five roosters at 3.5 (two roosters) or 4 (three roosters) days after a single administration of DES (25 mg/kg). Samples (1 mg of protein/0.4 mL) were adjusted to 0.02 M sodium phosphate (pH 7), 3% $NaDodSO_4$, 100 μg/mL $PhCH_2SO_2F$, and chromatographed on Sepharose 6B (0.9 × 20 cm) equilibrated with 0.02 M sodium phosphate (pH 7), 1% $NaDodSO_4$, 100 μg/mL $PhCH_2SO_2F$. Protein was measured in column fractions, and the distribution of recovered protein between the apo-VLDL-B and apo-VLDL-II fractions was calculated. [b] This value is the recovery of protein in the apo-VLDL-B and apo-VLDL-II fractions as a percent of total VLDL protein applied to the columns.

Upon electrophoresis of the cross-linked phosphorylase A, four bands were observed, the mobilities of which yielded a straight line when plotted against the logarithms of multiples of the subunit molecular weight. The mobilities of myosin, β-galactosidase, and serum albumin also fell on this standard curve at the expected positions (data not shown). For the estimation of apo-VLDL-B molecular weight, samples from seven animals were run in system A with 5 and 10 μg of protein per sample to minimize band spreading. This analysis yielded a molecular weight estimate of 350000 ± 5000 (±SD) for apo-VLDL-B.

Characteristics of Rooster Apo-VLDL-II. The results of several experiments in the present study indicate that the low molecular weight protein bands of rooster VLDL (Figure 1, lanes 3 and 4) are the same protein as previously described hen apo-VLDL-II. The electrophoretic mobilities of these bands (Figure 1, lane 4) in relation to low molecular weight standards are essentially the same as reported for hen apo-VLDL-II (Chan et al., 1976). Electrophoretic analysis of plasma VLDL as described in Figure 1 (lane 4) has shown variation in the proportions of these bands from sample to sample. This behavior is consistent with the formation of dimers through disulfide linkage as described for hen apo-VLDL-II (Chan et al., 1976). To test this point in more detail, [^{3}H]apo-VLDL-II was isolated by gel filtration in $NaDodSO_4$, S-carboxymethylated, and examined by electrophoresis in system B. The reduced sample showed radioactivity at the positions of both low molecular weight bands, while the reduced and S-carboxymethylated sample showed radioactivity only in the smaller of the two bands (data not shown). This analysis was also performed with unlabeled apo-VLDL-II in which case cylindrical gels were run and scanned at 280 nm subsequent to electrophoresis. These results also showed conversion of the larger band to the smaller band upon S-carboxymethylation. The electrophoretic mobilities of these bands and their behavior upon reduction and S-carboxymethylation are essentially the same as described for hen apo-VLDL-II (Chan et al., 1976).

The quantitative protein composition of rooster VLDL was determined by gel filtration chromatography in $NaDodSO_4$ as described in Figure 7. For this purpose plasma VLDL was isolated by flotation from five animals at either 3.5 or 4 days after a single administration of DES (25 mg/kg). As shown in Table I, apo-VLDL-II and apo-VLDL-B represented 46% and 54%, respectively, of total VLDL protein. This value for rooster apo-VLDL-II is essentially the same as found previously (Chan et al., 1976; Chapman et al., 1977) for the low molecular weight fractions of hen VLDL.

Discussion

The principal finding in this study is the occurrence of a single high molecular weight apoprotein when VLDL is isolated from rooster plasma in the presence of a serine protease inhibitor (Figure 1, lane 1). When isolated from serum without protease inhibition, VLDL contains multiple high molecular weight apoproteins (Figure 1, lane 2). Immunological analyses indicate that the multiple apoproteins of serum VLDL are quantitatively (Figures 3 and 4) and qualitatively (Figure 5) indistinguishable from plasma apo-VLDL-B. These data provide strong evidence that the multiple high molecular weight apoproteins of serum VLDL arise through proteolytic cleavage of apo-VLDL-B. The cleavage of apo-VLDL-B appears to occur in a qualitatively reproducible fashion in sera from different animals (Figures 1 and 6). In each case examined, a major cleavage product is seen at a molecular weight of approximately 290 000 (Figure 6, band 2). This component appears to persist as the major serum VLDL protein even when only a small quantity of intact apo-VLDL-B remains. This result suggests that band 2 is relatively resistant to further cleavage. Interestingly, a major serum VLDL protein corresponding to the molecular weight difference between apo-VLDL-B and band 2 (approximately 60 000) has not been observed in systems A or B (Figure 1, lanes 2 and 3) or in 10% polyacrylamide gels. The absence of this band may be explained if the 60 000 mol wt fragment were completely digested or randomly cleaved to a spectrum of minor bands which escaped detection. Alternatively, this component may be lost from the VLDL particle during isolation.

The occurrence of a single apo-VLDL-B species in plasma VLDL has permitted examination of some properties of this protein. Apo-VLDL-B was found to be stable to repeated gel-filtration chromatography in $NaDodSO_4$ and showed no significant breakdown following extraction with organic solvents. When examined by electrophoresis in system A, the mobility of apo-VLDL-B was the same with or without prior reduction. Similarly, reduction and S-carboxymethylation failed to significantly alter the electrophoretic mobility of apo-VLDL-B and failed to show detectable association between apo-VLDL-B and apo-VLDL-II through disulfide linkage (Figure 8). These data indicate that in the presence of $NaDodSO_4$ apo-VLDL-B behaves as a stable high molecular weight protein which shows no significant aggregation or intermolecular associations through disulfide linkage.

Electrophoretic analysis yielded a molecular weight estimate of 350 000 for apo-VLDL-B. While this estimate is reasonably precise, its accuracy depends on two basic assumptions. The first concerns the mobilities of the phosphorylase A multimers in relation to their actual molecular weights. Since the sites and number of interchain cross-links are unknown, there is uncertainty as to the shapes and $NaDodSO_4$ binding properties of the multimers. The assumption that the multimer mobilities approximate the mobilities of equivalent linear molecules is supported by the observation that the phosphorylase dimer runs at the expected position in relation to myosin and other standard proteins. Similarly, the mobilities of the trimer and tetramer lie on the standard curve extrapolated beyond the myosin standard. The estimate of apo-VLDL-B molecular weight with this method, therefore, is the same whether determined by interpolation from the multimer mobilities or by extrapolation from the mobilities of the other standard proteins. The second assumption concerns the $NaDodSO_4$ binding properties of apo-VLDL-B and the possible influence that associated carbohydrate may have on the electrophoretic mobility. These properties of apo-VLDL-B have yet to be sufficiently examined to evaluate their influence on the molecular weight estimate.

Electrophoretic analysis of plasma VLDL from the estrogen-treated rooster (Figure 1, lane 4) shows a low molecular weight protein with properties similar to those of apo-VLDL-II isolated from the laying hen. Rooster apo-VLDL-II shows two bands with the same, or very similar, mobilities as hen apo-VLDL-II (Chan et al., 1976). The behavior of these bands following reduction and S-carboxymethylation was also found to be the same as reported for hen apo-VLDL-II (Chan et al., 1976). In addition, rooster apo-VLDL-II was found to represent 46% of total VLDL protein (Table I). This value is essentially the same as reported for hen apo-VLDL-II (Chan et al., 1976; Chapman et al., 1977). These data strongly suggest that the rooster apo-VLDL-II described here is the same protein as hen apo-VLDL-II.

Chromatographic analyses of rooster VLDL showed that apo-VLDL-B and apo-VLDL-II represent 54% and 46%, respectively, of total VLDL protein (Table I). The recoveries of total protein in these analyses are sufficient to conclude that apo-VLDL-B and apo-VLDL-II are the major and possibly the only apoproteins of rooster VLDL. On the basis of this composition, the apo-VLDL-B molecular weight of 350 000 and the apo-VLDL-II molecular weight of 9444 (Jackson et al., 1977), the molar ratio of the apo-VLDL-II monomer to apo-VLDL-B is estimated to be approximately 32. It should be noted that this determination applies to VLDL obtained at a single time after estrogen treatment. At present, little is known about the proportions of these apoproteins in the basal state, at different times after estrogen treatment, or under the influence of other humoral agents. Similarly, the molar ratio of 32 is an average value that says nothing about the distribution of these apoproteins among different VLDL particles. Experiments directed toward these points are in progress and will be of importance in elucidating both the structure and regulation of the chicken VLDL particle.

The relationship of the results presented here to previous studies of chicken VLDL are of interest. In general, the high molecular weight protein fraction of VLDL from the laying hen has been found to be very heterogeneous. Electrophoretic analyses in a phenol–urea–acetic acid system (Hillyard et al., 1972; Hearn & Bensadoun, 1975) or in the presence of $NaDodSO_4$ (Holdsworth et al., 1974; Chapman et al., 1977) have shown as many as nine high molecular weight VLDL apoproteins. In one study of hen serum VLDL (Chapman et al., 1977), $NaDodSO_4$ gel electrophoresis showed seven or more protein bands ranging in apparent molecular weight from 140 000 to over 300 000. In the present study, hen plasma VLDL showed a single high molecular weight protein with the same electrophoretic mobility as apo-VLDL-B from the estrogen-treated rooster. Hen serum VLDL, on the other hand, showed multiple high molecular weight proteins. The implication from these data is that hen apo-VLDL-B is cleaved in the absence of protease inhibition in much the same fashion as apo-VLDL-B prepared from the serum of the estrogen-treated rooster. These results suggest that the marked heterogeneity of chicken VLDL apoproteins as previously reported may be a reflection of proteolytic cleavage during isolation. Consistent with this point is that protease inhibitors have not been employed in previous studies of chicken VLDL.

A possible exception to the marked heterogeneity of high molecular weight VLDL apoproteins has been observed with VLDL secreted from chick liver cells maintained in a monolayer culture (Tarlow et al., 1977). In this case, newly

secreted VLDL showed one predominant and several minor high molecular weight apoproteins. The predominant apoprotein was characterized as having a molecular weight of approximately 300 000 (Tarlow et al., 1977). At present, it is not possible to tell whether this 300 000 mol wt apoprotein corresponds to apo-VLDL-B or to the major 290 000 mol wt cleavage product observed in serum VLDL (Figure 6, band 2).

A point of interest in relation to the proteolytic cleavage of rooster apo-VLDL-B concerns recent evidence that VLDL may play a role in blood coagulation. Human and rabbit VLDL (Bajaj et al., 1976; Ploplis et al., 1977), for example, appear to serve as lipid sources in the activation of prothrombin by factor Xa under some in vitro reaction conditions. Similarly, human VLDL decreases the partial thromboplastin clotting time in recalcified platelet free human plasma (Vijayagopal & Ardlie, 1978). It has been proposed that some of the coagulation reactions may occur on the surface of VLDL particles under these conditions (Bajaj et al., 1977). If a similar phenomenon occurs in rooster blood, coagulation proteases localized to the VLDL particle could explain the marked cleavage of apo-VLDL-B when prepared from serum.

Acknowledgments

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Lac UV5 Transcription in Vitro. Rate Limitation Subsequent to Formation of an RNA Polymerase–DNA Complex†

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ABSTRACT: The kinetics of transcription of *lac* UV5 mRNA using purified DNA restriction fragment as template has been studied. This template, which contains only 203 base pairs, directs the formation of a 67-base *lac* mRNA with high specificity. The half-time for formation of a DNA–RNA polymerase complex is approximately 0.2 min. However, upon addition of 200 μM nucleoside triphosphates to this complex, RNA production proceeds with a half-time of approximately 1 min. Therefore, it is suggested that the rate-limiting step for *lac* UV5 mRNA production, under typical in vitro conditions, occurs subsequent to the formation of a promoter-specific complex.

The extent of specific operon expression in bacteria is largely determined by control at the level of initiation of transcription. Changing metabolic conditions may lead to the altered availability of regulatory factors which enhance or diminish the rate of production of specific mRNAs. These factors modulate the intrinsic efficiency of operon promoters which depends in an unknown manner on DNA sequence. Therefore, the mechanism by which DNA sequence directs RNA polymerase to initiate specific transcripts has received much attention [for a review, see Chamberlin (1976)].

Most current models (Pribnow, 1975; Seeburg et al., 1977; Chamberlin, 1976) suggest that regulation occurs primarily during the formation of a specific complex between RNA polymerase and promoter DNA. The evidence for this is as follows. Observed rates of complex formation for T7 (Hinkle & Chamberlin, 1972) and *lac*[1] DNA (Majors, 1977) are

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