Characterization of Apolipoproteins in Chicken Serum and Egg Yolk*

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ABSTRACT: Chicken serum and egg-yolk lipoproteins, isolated by ultracentrifugal procedures, were examined by (a) immunochemical methods, (b) disc gel and Agarose gel electrophoresis, (c) analytical ultracentrifugation, and (d) amino acid and end-group analyses. By these criteria, the VLDL isolated from egg yolk was identical with VLDL isolated from serum. Ultracentrifuge studies have shown that the very low density lipoproteins (VLDL) in chicken serum has a flotation rate of about S_f 40 and the low density lipoproteins (LDL) of about S_f 13. During Agarose gel electrophoresis, high density lipoproteins (HDL) migrated at the trailing edge of albumin, VLDL migrated only a short distance from the origin and LDL migrated slightly further than VLDL. The amino acid composition of the apolipoproteins in serum VLDL, egg-yolk VLDL, and serum LDL were similar and all had lysine at the N terminus and tyrosine at the C terminus. HDL apolipoprotein had aspartic acid at the N terminus and alanine and leucine at the C terminus. Approximately 90% of both VLDL and LDL apolipoproteins were accounted for by a polypeptide fraction which had a molecular weight of about 21,500 as determined by disc gel electrophoresis. This same polypeptide, when S-carboxymethylated, had a molecular weight of about 9000. The remainder of the protein in the VLDL apolipoprotein fraction could be separated into three or four bands by electrophoretic methods and had molecular weights ranging from 51,200 to 70,700. When examined by immunochemical methods, only one identical antigen was found in both VLDL and LDL, whereas another was found in HDL. On the basis of end-group analyses and disc gel electrophoresis, the possible existence of more than one polypeptide in each apolipoprotein fraction is inferred.

It has been recognized for many years that the lipid transport systems in avian species differs from that in mammals (Flock and Bollman, 1944; Taurog *et al.*, 1944). In mammals, exogenous fat is transported in chylomicrons *via* lymph, whereas in chickens chylomicrons are not formed and exogenous fat is transported in VLDL[1] *via* the portal vein (Noyan *et al.*, 1964). In the hen, serum VLDL is increased from a trace up to about 2000 mg/100 ml of serum with the onset of egg production (Schjeide, 1954). In the cockerel, VLDL can reach levels of 20,000 mg/100 ml of serum following stilbestrol treatment (Hillyard *et al.*, 1956). On the basis of these observations, the chicken would appear to be a good model for studies on the mechanism of lipid transport. However, at present, only fragmentary information is available on the nature of the apolipoproteins in chicken serum lipoproteins. The results of studies on the characterization of the apolipoproteins in chicken serum and egg yolk are presented here.

Experimental Section

Source of Serum. Blood was obtained from fed adult White Leghorn cockerels and laying hens through the courtesy of the New California Poultry Co., Oakland, Calif. The sera from 12–18 chickens were pooled and the lipoproteins were usually isolated immediately. However, when delay was necessary, the sera was stored at 4° with 0.01% merthiolate.

Preparation of Lipoproteins. Serum lipoprotein fractions (VLDL, LDL, and HDL) were isolated at 17° in a 40.3 rotor with a Beckman-Spinco Model L-HV preparative ultracentrifuge by a modification of the procedures of Ewing *et al.* (1965). Each centrifugation was extended to 42 hr since the usual 18- to 24-hr centrifugations resulted in cross contamination of apolipoproteins as determined by immunodiffusion.

Egg yolk was diluted with three volumes of a NaCl solution (1.006 g/ml) and the yolk granules removed by centrifuging for 30 min at 15,000*g*. The protein concentration of the supernatant solution was adjusted to about 10 mg/ml and the Ey-VLDL isolated by the same method used for serum VLDL.

Lipid Extraction. Two methods of extraction were used. In the first, the lipoprotein solution was dialyzed against distilled water at 4° for 24–48 hr. The dialyzed solution was lyophilized and the residue allowed to stand overnight at room temperature with 20 volumes of ethanol–acetone (1:1, v/v) and then extracted with ethanol–diethyl ether–chloroform (6:2:1, v/v). The residue was washed with diethyl ether and acetone, air-dried, and stored *in vacuo*. Delipidized lipoproteins (apolipoproteins) prepared by this method were used for all experiments except as noted. In the second method, lipids were extracted by heating the lipoprotein solution twice for 1-hr periods at 60° with 20 volumes of ethanol–diethyl ether–chloroform (6:2:1, v/v). The residue was washed with acetone, suspended in 5% trichloroacetic acid, and heated for 30 min at 80° to remove adsorbed free amino acids. The protein was washed with diethyl ether and acetone, air-dried, and stored *in vacuo*. Lipoproteins delipidized by this second method were only used for amino acid end-group analyses.

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[1] Abbreviations used are: VLDL, very low density lipoproteins; Ey-VLDL, egg yolk very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, lipid-free apolipoprotein; RCM, reduced and carboxymethylated; CA, carbamylated; DNP, dinitrophenyl; FDNB, 1-fluoro-2,4-dinitrobenzene.

Preparation of Derivatives: Carbamylation, Carboxymethylation, and Succinylation. Native lipoproteins were carbamylated by reacting equal volumes of lipoprotein solution with 2 M potassium cyanate in 0.1 M Tris-HCl buffer (pH 8.2). The mixture was allowed to stand at room temperature overnight. In the case of apolipoproteins, reduction prior to carbamylation facilitated solubilization of the protein so that complete carbamylation could be achieved. Thus, carbamylation was carried out in a reducing mixture consisting of 6 M guanidine hydrochloride, 0.05 M *N*-ethylmorpholine acetate (pH 8.2), 0.2 M β-mercaptoethanol, and 0.4 M potassium cyanate. All carbamylated preparations were dialyzed overnight against appropriate buffers before use.

Reduction and carboxymethylation of the apolipoproteins was carried out by the method of Hirs (1956) and succinylation by the method of Klotz (1967).

Immunochemistry. All native lipoprotein solutions were dialyzed for 24 hr against 0.15 M NaCl. Apolipoproteins were dissolved in 0.05 M Tris-HCl buffer (pH 8.2) containing 0.05 M sodium decyl sulfate (Brown *et al.*, 1969). The detergent concentration was reduced by dialysis against 0.05 M Tris-HCl buffer (pH 8.2) containing 0.001 M sodium decyl sulfate for 24 hr. After dialysis, the protein concentration was adjusted to 5 mg/ml.

Antisera were produced in New Zealand white rabbits (1.5–2.5 kg) by subcutaneous injections at 2-week intervals of lipoprotein solutions emulsified with equal volumes of complete Freund's adjuvant (Difco Laboratories). From 1 to 3 mg of VLDL and LDL protein was injected each time (total, 4–12 mg) and 4–15 mg of HDL protein was injected each time (total, 16–60 mg). The rabbits were bled from the marginal ear vein 7 days after the third or fourth injection and the sera obtained was stored frozen in small aliquots in the presence of 0.01% merthiolate. Chicken serum HDL was markedly less antigenic than either VLDL or LDL.

Ouchterlony double diffusion (Ouchterlony, 1949) was carried out in 1 × 3 in. microscope slides coated with 0.6% Agarose gel in 0.05 M barbital buffer (pH 8.6). Diffusion was carried out at room temperature in a humid chamber usually for 24 hr and, on occasions, for periods up to 7 days. The gel was washed for 3 days with 0.15 M NaCl and 2 hr with distilled water. The slides were air-dried and stained with 1% aniline blue black in 2% acetic acid and destained with 2% acetic acid.

Immunoelectrophoresis (Scheidegger, 1955) was carried out in 0.6% Agarose gel in 0.05 M barbital buffer (pH 8.6) for three hours at 4 mA/slide. The antisera was then placed in the trough and allowed to diffuse for 24–48 hr at room temperature. The gel was washed and stained by the procedures given above.

N-Terminal Amino Acids. A modification of the FDNB method was used (Biserte *et al.*, 1960). Delipidated lipoprotein (2–5 mg) was dissolved in 1 ml of 2% $NaHCO_3$ containing sodium decyl sulfate (2 mg for HDL and 26 mg for VLDL and LDL); 1.5 ml of 95% ethanol and 0.02 ml of FDNB were added and the mixture was shaken in the dark at room temperature for 2 hr. The mixture was acidified with 0.1 ml of 5 N HCl and the precipitated DNP-protein was washed three times with ethanol and three times with acetone. The air-dried DNP-protein was hydrolyzed with 1 ml of 6 N HCl in a sealed tube at 100° for 16 hr. The hydrolysate was diluted with 5 ml of distilled water and extracted five times with 2-ml aliquots of diethyl ether. The combined ether extracts were washed in 0.1 N HCl, concentrated to dryness, and the dinitrophenol was removed by sublimation. The ether extracted aqueous phase was passed through a 5-g Florisil (60–100 mesh) column (1 × 11 cm) and the free amino acids eluted with 50 ml of 1 N HCl. The water-soluble DNP-amino acids were eluted from the column with 10–12 ml of ethanol–1 N HCl (4:1, v/v). The eluate containing the DNP-amino acids was taken to dryness *in vacuo* and the residue was again reacted with FDNB to convert ϵ-DNP-lysine, *O*-DNP-tyrosine, and imidazole-DNP-histidine to the di-DNP derivatives. When α-DNP-arginine is present, these di-DNP derivatives can be separated from α-DNP-arginine by extraction with diethyl ether and ethyl acetate (Rovery *et al.*, 1953).

The ether-soluble DNP-amino acids were separated and identified by two thin-layer chromatographic methods. The first system used silica gel G (0.25 mm thick) with *tert*-butyl alcohol–methyl ethyl ketone–1.6 N ammonia (2:2:1, v/v) as developing solvent in the first dimension and chloroform–benzyl alcohol–acetic acid (80:20:3, v/v) in the second dimension. The second system used microcrystalline cellulose (0.5 mm thick) and 0.25 M sodium phosphate (pH 6.1) as developing solvent in the first dimension and isobutyl alcohol–ethanol–0.01 M sodium phosphate, pH 6.0 (5:3:2, v/v) in the second dimension.

The water-soluble DNP-amino acids were separated on microcrystalline cellulose (0.5 mm thick) by two-dimensional chromatography using two different solvent systems. The first solvent pair used was *tert*-butyl alcohol–methyl ethyl ketone–1.6 N NH_4OH (2:2:1, v/v) and *n*-propyl alcohol–methyl ethyl ketone–1.6 N NH_4OH (2:2:1, v/v), and the second solvent pair was *n*-propyl alcohol–methyl ethyl ketone–1.6 N NH_4OH (2:2:1, v/v) and chloroform–ethanol–acetic acid (10:10:1, v/v).

C-Terminal Amino Acids. Both carboxypeptidase digestion and hydrazinolysis were used to determine the C-terminal amino acids. Solutions containing either native lipoproteins or dilipidated lipoproteins (2–3 mg of protein/ml) in 0.2 M *N*-ethylmorpholine acetate (pH 8.2 and 8.9) and in 0.2 M ammonium acetate (pH 6.0 and 6.7) were incubated with carboxypeptidase A dissolved in 0.2 M NH_4HCO_3 (Ambler, 1967). Enzyme/substrate ratios from $^1/_{20}$ to $^1/_{300}$ were employed at room temperature (22°) and at 37° for time intervals from 1 min to 24 hr. For carboxypeptidase B digestion, the enzyme was incubated with lipoprotein preparations in 0.2 M *N*-ethylmorpholine acetate (pH 8.2). All enzyme reactions were stopped with 0.1 ml of 8 N acetic acid/ml of reaction mixture and the insoluble protein removed by centrifugation. The supernatant was either taken to dryness and used directly for amino acid analysis or the amino acids were recovered by batchwise adsorption on Dowex 50-X8 (20–50 mesh) and elution with 2 M NH_4OH (Ambler, 1967).

For hydrazinolysis, delipidated lipoprotein (10–15 mg) was dissolved in 0.5 ml of anhydrous hydrazine, sealed under vacuum in the apparatus described by Schaffer *et al.* (1966), and heated to 100° for various time intervals from 6 to 48 hr (Fraenkel-Conrat and Tsung, 1967). Excess hydrazine was removed by lyophilization and the residue was dissolved in 3 ml of water. The hydrazides were reacted with and extracted by 0.2 ml of heptanal (Dus *et al.*, 1962). The heptanal extraction was repeated twice and the excess heptanal removed by three washings with ethyl acetate. The aqueous phase was taken to dryness *in vacuo* over P_2O_5 and concentrated H_2SO_4 for subsequent amino acid analysis.

Amino Acid Analyses. The amino acid composition of apolipoproteins was determined by the procedure of Benson and Patterson (1965) with a Beckman amino acid analyzer.

Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1956) and tryptophan was estimated by the spectrophotometric procedure of Edelhoch (1967). These analyses were performed by the AAA Laboratory, Seattle, Wash.

The amino acids liberated by carboxypeptidase digestion and by hydrazinolysis were usually determined by a combined high voltage electrophoresis–thin-layer chromatographic method, although in some cases they were quantitated with the amino acid analyzer. The glutamine and asparagine contents of the carboxypeptidase digests was determined as given above by the change in glutamic and aspartic acid concentrations before and after hydrolysis with 2 N HCl for 2 hr at 100°. Separation of the amino acids was carried out on MN300 cellulose (0.25 mm thick) on 20 × 40 mm glass plates. Electrophoresis was performed in the long dimension on a Brinkman high-voltage pherograph for 1 hr at 1750 V, 15–20 mA, with 4% formic acid (pH 2.0) as electrolyte. Thin-layer chromatography was next carried out in the short dimension with isopropyl alcohol–water–formic acid (80:20:4, v/v). Amino acids were detected with the ninhydrin–cadmium acetate reagent of Atfield and Morris (1961).

Peptide Maps. VLDL and HDL apolipoproteins were oxidized with performic acid (Hirs, 1956). The oxidized product (5 mg) was digested with 0.1 mg of trypsin in 1 ml of 0.2 M NH_4HCO_3 for 18 hr at 37°. The reaction mixture was acidified with acetic acid, heated for 10 min at 90°, and centrifuged. The supernatant fraction was taken to dryness in a small silicone-treated crucible *in vacuo* at 40°.

The peptides in the residue were dissolved in a small volume of water and separated on MN300 cellulose (0.25 mm thick) on 20 × 40 cm glass plates. Electrophoresis was first performed in the long dimension on a Brinkman high-voltage pherograph for 1 hr at 1750 V, 15–20 mA, with 4% formic acid, pH 2.0, as electrolyte. Chromatography was next carried out in the short dimension. The solvent system for the VLDL peptides was isopropyl alcohol–water–formic acid (80:20:4, v/v) and for the HDL peptides, isobutyl alcohol–pyridine–water (35:35:30, v/v). Peptides were detected with ninhydrin by the method of Atfield and Morris (1961).

Molecular Weight Determination by Disc Gel Electrophoresis. Disc gel electrophoresis was carried out with the phenol–urea–acetic acid system of Takayama *et al.* (1966), with 5, 7.5, and 10% gels made from a stock solution of *N,N'*-methylenebisacrylamide–acrylamide (1:37.5, v/v). Cytochrome *c* was added to each sample and the relative mobility (R_m) of each protein fraction calculated as a percent of the cytochrome *c* migration. Molecular weight was determined by the method of Hedrick and Smith (1968) whereby per cent gel concentration is plotted against 100 log R_m for each protein and the slope determined (100 log R_m/% gel). Molecular weight and slope data were fitted to the curve $y = \alpha + \beta x$ by the method of least squares, where y is molecular weight and x is slope. With the seven reference proteins used, constants α and β were determined to be 12.72 and 10.36, respectively. When insulin, after reduction with β-mercaptoethanol, was used as a reference protein, a slope of -1.08 was obtained. Although this value does not deviate appreciably from the curve constructed from the slopes of the other reference proteins, the molecular weight of reduced insulin calculated from these data was significantly different from literature values (about 2900). Therefore, insulin was not used as one of the reference proteins.

Agarose Gel Electrophoresis. When needed to provide optimal conditions, some fractions were concentrated by ultrafiltration in a Centriflo membrane cone (Amicon Corp.). Electrophoresis of serum and isolated lipoprotein fractions was performed on 0.5% Agarose gel on 1 × 3 in. microscope slides in 0.05 M barbital buffer (pH 8.6) for 2 hr at 4 mA/slide. Samples (2–4 μl) were placed in slots, 1 cm long, cut with the edge of Whatman No. 50 filter paper. The Agarose gel was fixed in 5% acetic acid in 60% methanol, dried at room temperature, and stained with 1% aniline blue black in 2% acetic acid.

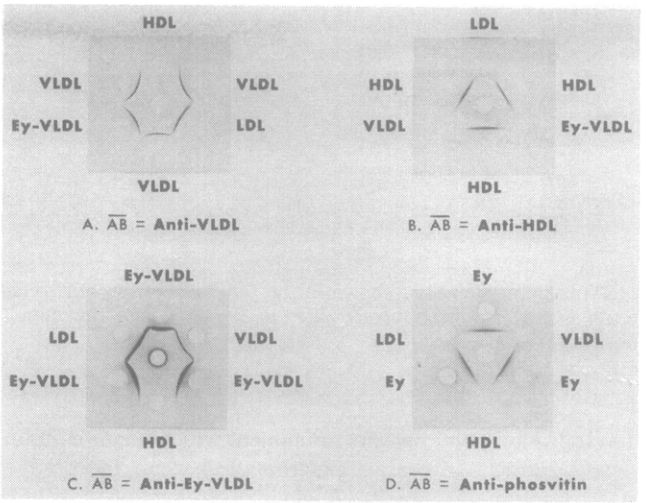

FIGURE 1: Immunodiffusion patterns of chicken serum and egg yolk lipoproteins. The antiserum ($\overline{AB}$) was placed in the center well in each experiment. Egg yolk (Ey), diluted with 0.15 M NaCl, was used as the source of phosvitin.

Ultracentrifuge Study. The methods of Ewing *et al.* (1965) were used for (a) the analytical ultracentrifuge characterization of serum VLDL, LDL, and HDL from laying hens, and (b) for the computer analysis of the results. These procedures were performed in the Donner Laboratory, University of California, Berkeley, Calif.

Materials. The following items were purchased from Sigma Chemical Co.: carboxypeptidases A and B both treated with diisopropyl fluorophosphate, bovine pancreas trypsin treated with diphenylcarbamyl chloride, horse heart cytochrome *c*, soybean trypsin inhibitor, bovine pancreas chymotrypsin, ovalbumin, and DNP-amino acid standards. Rabbit anti-phosvitin serum and bovine pancreas insulin were obtained from Calbiochem and sperm-whale myoglobin from Mann Research Laboratory. Anhydrous hydrazine (97%+) was obtined from Matheson Coleman & Bell and used without further treatment. Silica gel G, NM300 cellulose, and microcrystalline cellulose (Avicel) were purchased from Brinkmann Instruments and Florisil from the Floridin Co.

Results

Immunochemical Analyses. The precipitin arcs which developed with VLDL, LDL, and Ey-VLDL, that had reacted with anti-VLDL serum and anti-Ey-VLDL serum, fused without spur formation, suggesting the complete identity of the apolipoproteins in these three lipoprotein fractions (Figure 1A, C). VLDL and LDL were essentially free of HDL apolipoprotein (Figure 1B). All lipoprotein fractions studied here were free of phosvitin as determined by double diffusion against anti-phosvitin serum (Figure 1D). Immunoelectrophoresis of VLDL and LDL resulted in single precipitin lines (Figure 2). As a result of storage at 2°, chicken HDL

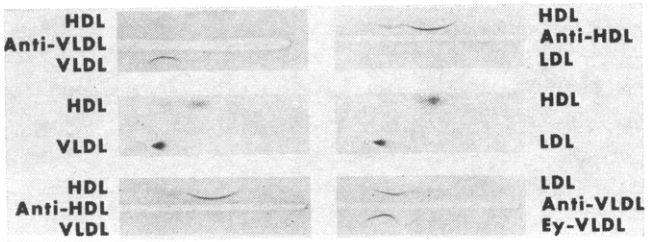

FIGURE 2: Immunoelectrophoresis patterns of chicken serum and egg-yolk lipoproteins are shown in the top and bottom horizontal rows. Control electrophoresis patterns are shown in the middle horizontal row.

developed a second protein component which migrated more slowly during Agarose gel electrophoresis than the original HDL fraction (Figure 2). A similar observation has been made with human HDL (Levy and Fredrickson, 1966). However, in the case of the chicken, both HDL components react with anti-HDL serum yielding two precipitin arcs which fuse (Figure 2). Although these results do not prove the existence of two antigens in the HDL preparations, they do not preclude such a possibility.

When anti-LDL serum was absorbed with both VLDL and HDL to remove antibodies specific for their apolipoproteins, no other antibody was found which reacted with LDL. In order to determine if masked antigens were present, immunochemical studies were carried out with lipid-free apolipoproteins and their derivatives (Figure 3). Carbamylation of apoVLDL and apoHDL increased solubility in dilute buffers without effecting antigenicity. Although succinylation increased solubility, it destroyed antigenicity. Electrophoresis of RCM-apoVLDL and apoHDL, followed by diffusion against either anti-apoVLDL or anti-apoLDL, resulted in the development of a single precipitin line for RCM-apoVLDL and none for apoHDL (Figure 3). ApoHDL gave a single precipitin line with anti-apoHDL while no precipitin line was found with RCM-apoVLDL (Figure 3). None of the other apolipoprotein preparations studied (apoLDL, apoVLDL, and CA-apoVLDL) developed more than one precipitin line. Nevertheless, minor polypeptides may not have been detected in apoVLDL and apoLDL by immunochemical methods due to (a) the loss of these polypeptides during apolipoprotein preparation, (b) low antigenicity, or (c) masking by the major polypeptides.

Terminal Amino Acids. Lysine was the only N-terminal amino acid detected by the FDNB method in the apolipoproteins of LDL, VLDL, and Ey-VLDL (Table I). Following hydrazinolysis, tyrosine was the major free amino acid recovered from VLDL. In addition to tyrosine, carboxypeptidase A treatment released valine and leucine however the sequence of their release was not determined.

Neelin and Cook (1961) reported the presence of lysine and arginine at the N terminus and glutamic acid at the C terminus of α-lipovitellin (LDL), β-lipovitellin (LDL), and lipovitellenin (VLDL) isolated from egg yolk. After an extensive search for α-DNP-arginine in the products of dinitrophenylation, we have concluded that arginine is not an N-terminal amino acid in either chicken serum VLDL or Ey-VLDL apolipoproteins and that lysine is the major N-terminal amino acid. No glutamic acid was found as a C-terminal amino acid in apoLDL, apoVLDL, or Ey-apoVLDL by the carboxypeptidase method (ten determinations each).

The major N-terminal amino acid of HDL apolipoprotein is aspartic acid (Table I). Alanine and leucine were both found after hydrazinolysis indicating the presence of two C-terminal amino acids in HDL apolipoprotein. Alanine, valine, and serine were the only amino acids released from delipidated HDL by the action of carboxypeptidase A at pH 6.0, 6.7, and 8.2. The release of amino acids studied at room temperature and pH 8.2 for various time intervals revealed that alanine appeared after 2–5 min, valine appeared somewhat later and serine after 10–30 min. These results suggest the sequence alanine-valine-serine with alanine at the C terminus.

The quantitative recovery of amino acids from carboxypeptidase A digestion of apoHDL at pH values 6.7, 8.2, and 8.9 are given in Table II. At pH 6.7, approximately equal molar amounts of alanine, valine, and serine were released. At pH 8.9, leucine, glutamine, and lysine were released in addition

TABLE I: N- and C-Terminal Amino Acids of Chicken Serum and Egg-Yolk Apolipoproteins.

Apolipoprotein	Terminal Amino Acid	
	N	C
HDL	Asp	Ala
		Leu
LDL	Lys	Tyr
VLDL	Lys	Tyr
Ey-VLDL	Lys	Tyr

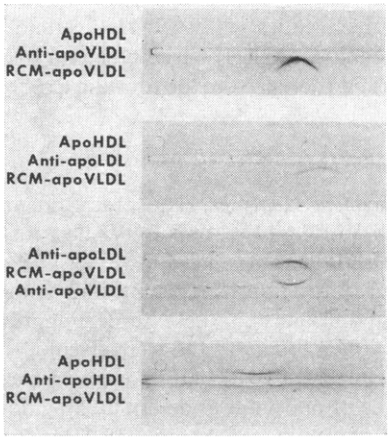

FIGURE 3: Immunodiffusion and immunoelectrophoresis patterns of chicken serum apolipoproteins and their derivatives.

TABLE II: Amino Acid Released from HDL Apolipoprotein by Carboxypeptidase A.

pH[a]	Moles of Amino Acid Released per 100,000 g of Protein					
	Ala	Val	Ser	Leu	Gln	Lys
6.7	2.95	3.07	3.11	0	0	0
8.2	2.96	2.79	1.43	0	0	0
8.9	1.87	2.22	Trace	1.81	2.11	1.18

[a] Each incubation was for 1 hr at 37° at the pH indicated.

TABLE III: Molecular Weight of Chicken Serum Apolipoproteins Determined by Disc Gel Electrophoresis.

Protein	Slope[a]	Molecular Weight			
		Lit. ($\times 10^3$)	App[b] ($\times 10^3$)	Deviation (%)	Reference
Standards					
Cytochrome *c*	0	12.4	12.7	2.4	Margoliash (1962)
Myoglobin, sperm whale	0.54	17.8	18.3	2.8	Edmunson and Hirs (1962)
Soybean trypsin inhibitor	0.90	22.6	22.0	2.7	Frattali and Steiner (1968)
Trypsin	0.98	23.8	22.9	3.8	Schachman (1963)
Chymotrypsin	1.32	25.3	26.4	4.3	Moroux and Rovery (1966)
Carboxypeptidase A	2.00	34.3	33.4	2.6	Schachman (1963)
Ovalbumin	3.16	45.0	45.5	1.1	Warner (1954)
Lipoproteins					
ApoVLDL, band 1	0.88		21.8		
ApoVLDL, band 2	3.71		51.2		
ApoVLDL, band 3	4.64		60.8		
ApoVLDL, band 4	5.60		70.7		
RCM-apoVLDL, band 1	−0.36		9.0		
ApoLDL, band 1	0.83		21.3		
ApoHDL, band 2	1.68		30.1		

[a] Average values for ten determinations. [b] Calculated from values for slope and the constants α and β by the procedures given in the text.

to alanine, valine, and serine. Lysine is probably not located at the C terminus since no basic amino acids were released by digestion of apoHDL with carboxypeptidase B. Leucine, glutamine, and lysine could be the continuation of the same peptide possessing the sequence alanine, valine, and serine. However, since free leucine was consistantly found after hydrazinolysis, and little or no serine was released at pH 8.9, leucine, glutamine, and lysine may be part of a second peptide.

Regardless of the method of delipidation, the resultant apolipoproteins gave the same results when subjected to end-group analyses.

The action of carboxypeptidase A on native VLDL did not produce any free amino acid. On the other hand, the pattern of amino acid release was the same for both native HDL and apoHDL, demonstrating that the C terminus in native HDL is available to carboxypeptidase A for cleavage.

Molecular Weight Determination. Disc electrophoresis patterns for apoVLDL, RCM-apoVLDL, apoLDL, and apoHDL are shown in Figure 4. A component with the same relative migration (band 1) accounted for 90% or more of the protein[2] for both apoVLDL and apoLDL. The molecular weight of the major component in apoVLDL was 21,800 and in apoLDL, 21,300 (Table III). In the reduced S-carboxymethylated form of apoVLDL, the molecular weight of this same component (band 1) was 9000. These results indicate that reduction and carboxymethylation cleaves the major protein constituent of apoVLDL into two polypeptides of approximately equal size.

The other protein components of apoVLDL separated into three components (bands 2, 3, and 4), with molecular weights of 51,200, 60,800, and 70,700 (Table III). A fifth band occasionally appeared. The molecular weights of each of these minor components did not change after reduction and carboxymethylation. Although minor bands appeared in apoLDL samples, they did not show the same pattern as in apoVLDL. The most consistently occurring minor band in apoLDL had a molecular weight of about 40,000.

HDL apolipoprotein was separated into five or more components by disc electrophoresis (Figure 4). The molecular weight of the fastest migrating major component (band 2) was 30,100 (Table III). One minor component (band 1) had a molecular weight of 20,000–24,000 but did not appear in

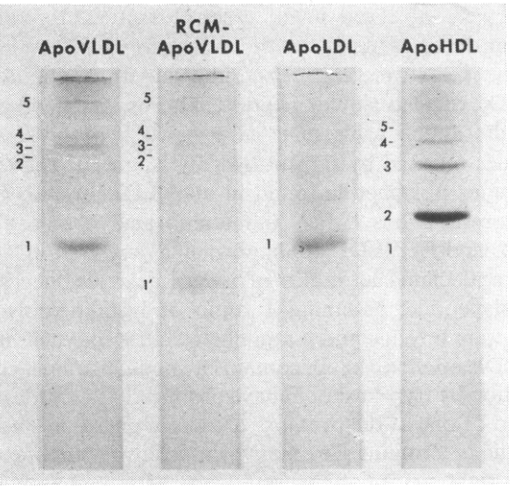

FIGURE 4: Disc gel electrophoresis patterns for chicken serum apolipoproteins. Electrophoresis was carried out with the phenol–urea–acetic acid system of Takayama *et al.* (1966) for 2.5 hr at 4 mA/tube in 7.5% acrylamide gels.

[2] The stained disc gels were scanned at 580 nm with a Beckman DU spectrophotometer fitted with a Gilford Model 2410 linear transport and automatically recorded. The distribution of each protein component was estimated as a percentage of the sum of the areas under the peaks.

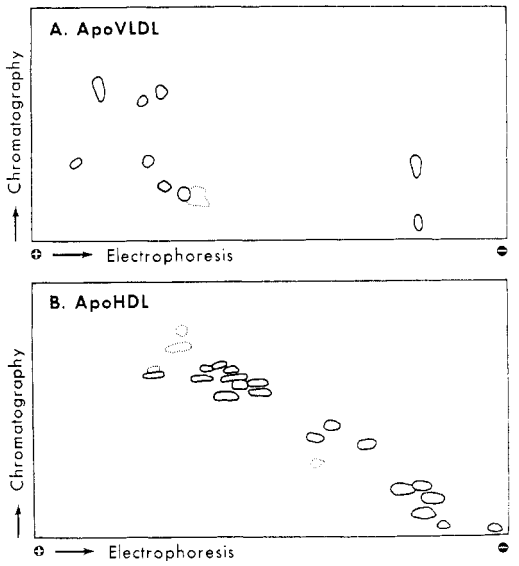

FIGURE 5: Tracings of peptide maps made from tryptic digests of (A) apoVLDL and (B) apoHDL.

all samples. When apoHDL or its derivatives were analyzed with disc gel systems at pH 8.8 containing (a) 8 M urea, (b) 1% Triton X-100, or (c) 1% sodium dodecyl sulfate, the patterns observed were not significantly different from those when phenol–urea–acetic acid was used.

Amino Acid Analyses. The amino acid composition of apoLDL, apoVLDL and Ey-apoVLDL are very similar (Table IV), consistent with the results obtained by immunochemical methods, disc electrophoresis and by N- and C-terminal amino acid analyses. The amino acid composition for egg-yolk vitellenin (Table IV, column 6) reported by Steer *et al.* (1968) is compared to the data reported here (Table IV, column 5) for Ey-apoVLDL. With the exception of tyrosine, most differences in amino acid concentration are minor. The amino acid composition of egg-yolk LDL obtained by Evans *et al.* (1968) and shown in Table IV, column 7 is also similar.

Peptide Maps. The performic acid oxidized VLDL apolipoprotein was completely digested by trypsin as little or no material remained at the origin after electrophoresis and chromatography. When six different apoVLDL preparations were digested with trypsin, the same nine ninhydrin-positive spots were observed in all cases (Figure 5A). With some preparations, as many as three additional faint ninhydrin-positive spots were seen. Experiments with tryptic digests of apoLDL yielded similar peptide patterns. We have assumed that the amino acids released by acid hydrolysis, and the peptides released by trypsin hydrolysis, are all derived from the major polypeptide found in apoVLDL by disc gel electrophoresis. Thus, when the amino acid composition for VLDL and Ey-VLDL apolipoproteins presented in Table IV was recalculated as moles of amino acid residue per mole of polypeptide, assuming 1 mole of histidine/polypeptide, there were 6 lysine and 4 arginine residues for each histidine. If VLDL apolipoprotein contains a single histidine, complete digestion by trypsin should have produced 11 peptides. Lysine should be one of the products of trypsin digestion since lysine is at the N terminus. The ninhydrin-positive component in the lower right corner of Figure 5A was identified as free lysine in the following manner. Only basic amino acids, or peptides containing basic amino acids, would migrate this far during high-voltage electrophoresis. However, this component did not contain arginine or histidine since it gave negative Sakaguchi and Pauly reactions (Block *et al.*, 1955). Chromatography of lysine with the trypsin digest resulted in the complete superimposition of this compound with lysine.

TABLE IV: Amino Acid Composition of Chicken Apolipoproteins.

Amino Acid	Moles of Amino Acid per 100 Moles Recovered[a]					
	Apo-HDL	Apo-LDL	Apo-VLDL	Ey-Apo-VLDL	Vitellenin[b]	LDL[c]
Lys	8.92	7.46	7.02	7.70	7.59	7.47
His	0.77	1.43	1.12	1.40	1.30	1.41
Arg	7.51	4.40	4.58	5.35	5.35	5.36
Asp	8.54	10.5	10.9	11.4	10.9	9.93
Thr	4.57	6.31	6.26	6.48	6.36	6.72
Ser	4.29	7.75	7.09	7.50	7.02	6.46
Glu	20.5	13.1	11.3	11.3	10.9	11.9
Pro	4.35	3.54	3.62	3.02	3.35	3.58
Gly	1.96	5.07	4.44	4.61	5.17	5.49
Ala	9.19	7.36	7.65	7.79	7.96	7.76
Half-Cys	0.23	0.80	0.99	1.01	0.80	
Val	5.34	6.22	6.60	6.73	6.91	7.25
Met	2.33	1.72	2.02	1.48	1.05	2.04
Ile	2.51	5.55	6.54	6.85	6.53	6.74
Leu	13.0	10.3	10.4	10.5	10.9	10.4
Tyr	2.55	2.39	3.41	2.12	3.90	3.37
Phe	2.07	4.40	4.25	4.19	3.98	4.11
Tryp	1.38	1.66	1.77	0.61		

[a] The values are given as averages of two closely agreeing determinations. [b] Data from Steer *et al.* (1968). [c] Calculated from data for egg yolk LDL reported by Evans *et al.* (1968).

The minimum molecular weight calculated from the amino acid composition is 8260 for Ey-VLDL apolipoprotein and 10,230 for VLDL apolipoprotein. These values are in the same range (9000) found by disc gel electrophoresis for the major polypeptide in VLDL apolipoprotein after reduction and carboxymethylation. It should be noted that there is one half-cystine for each mole of histidine. This relationship suggests that two polypeptides, each with a molecular weight of about 9000, are linked together by a single disulfide bond.

All of the ten peptide maps prepared from different tryptic digests of apoHDL gave similar patterns (Figure 5B). We have assumed that HDL apolipoprotein was homogeneous for the following calculation. When the amino acid composition for this protein is calculated as moles of amino acid residue per mole of polypeptide (assuming 1 mole of histidine per polypeptide), one finds 12 lysine and 10 arginine residues for each histidine. If the assumption is correct, then digestion by trypsin should have produced 23 peptides. From 20 to to 24 ninhydrin-positive spots were found for apoHDL (Figure 5B). The minimum molecular weight calculated on this basis is 15,430. Although the number of peptides observed are consistent with this minimum molecular weight, the validity of this calculation remains in doubt since we have not been able to establish the homogeneity of apoHDL.

Agarose Gel Electrophoresis. Chicken serum HDL migrated at the trailing edge of albumin in 0.5% Agarose gel (Figure 6).

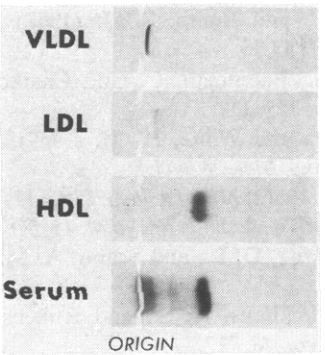

FIGURE 6: Agarose gel electrophoresis of chicken serum lipoproteins.

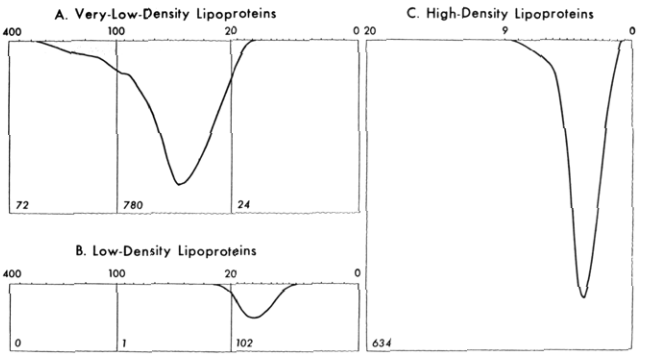

FIGURE 7: Analytical ultracentrifuge patterns of (A) VLDL, (B) LDL, and (C) HDL isolated from the serum of laying hens. VLDL and LDL flotation rates are in S_f units and HDL in $F_{1.20}$ units. The schlieren data were developed for graphic presentation by the computer program of the Donner Laboratory, University of California, Berkeley. The figure in the lower left corner of each box represents mg of lipoprotein/100 ml of serum in the flotation range given by the horizontal axis.

A minor component of the HDL moving more slowly than the major component was usually observed (Figures 2 and 6). VLDL migrated only a short distance from the origin and LDL migrated slightly further. When these isolated lipoprotein preparations were stored at 2° for 1 or 2 weeks an then subjected to electrophoresis, the LDL band appeared very diffuse, whereas the VLDL band remained sharp.

Analytical Ultracentrifugation. The computer-derived ultracentrifuge patterns for lipoproteins from the serum of laying hens (Figure 7) show two major species of lipoproteins, VLDL, and HDL. The VLDL peak position is about S_f 40 (Figure 7A) and the small LDL component has a peak position of about S_f 13 (Figure 7B). HDL has a single, sharp peak (Figure 7C). In man (Ewing *et al.*, 1965), β-lipoprotein with a peak position of about S_f 6 is the principal low-density lipoprotein class. In the cockerel and immature hen, VLDL is absent presumably due to the rapid clearing of VLDL from the bloodstream by lipoprotein lipase (Korn and Quigley, 1957).

Discussion

There are no lipoprotein classes above S_f 400 in chicken serum and there is little or no VLDL (S_f 20–400) in the serum of the immature hen or cockerel. It would appear, therefore, that the lipoprotein lipase activity of heart, adipose tissue, as well as other extraheptatic tissues (Korn and Quigley, 1957), is sufficient to prevent VLDL from accumulating in the blood of all nonlaying birds. The massive increase in VLDL at the onset of egg production in the hen is the result of an increase in synthesis by the liver (Florsheim *et al.*, 1963) mediated by elevated levels of estrogen. Thus, the accumulation of VLDL in serum serves as a reservoir of VLDL needed for egg-yolk production. The identity of Ey-VLDL with serum VLDL supports the view that serum VLDL is removed intact from the blood by the ovary in the laying hen (Sturkie, 1965).

In our investigations of the protein moieties of chicken lipoproteins, we found two N-terminal and three C-terminal amino acids, suggesting the presence of at least three polypeptides. However, by immunological methods, we could only detect two polypeptides. Our findings with chicken apolipoproteins differ from those obtained by others for human lipoproteins in which four N-terminal and six C-terminal amino acids were shown (Brown *et al.*, 1969, 1970; Shore and Shore, 1968, 1969). In line with these observations was the demonstration that four or more antigens are present in the serum lipoproteins of man (Brown *et al.*, 1969). Similar results were obtained with rats (Koga *et al.*, 1969; Kook *et al.*, 1971) and dogs (Solyom *et al.*, 1971). The data presented here support the view that chicken lipoproteins are less complex than those in mammals.

The immunological studies of Walton and Darke (1963) demonstrated that human low-density lipoproteins cross-react with LDL in ten other mammalian species, but failed to cross-react with chicken lipoproteins. We have shown that the apolipoproteins from chicken VLDL, LDL, and HDL isolated by ultracentrifugal methods have different N- and C-terminal amino acids than these same fractions prepared from human serum. These observations suggest that the apolipoproteins from avian and mammalian lipoproteins may not only be immunologically but also chemically nonidentical.

Results from N- and C-terminal amino acid analyses, peptide maps, amino acid composition and disc gel electrophoresis suggest that a single polypeptide with a molecular weight of about 9000 accounts for about 90% of the VLDL and LDL apolipoproteins. Since (a) there is only one half-cystine per mole of histidine, equivalent to a minimum molecular weight of about 9000, and (b) the major apoVLDL polypeptide has a molecular weight of about twice the minimum molecular weight, we propose that the VLDL apolipoprotein contains a subunit which may consist of two polypeptide chains linked together through a single disulfide bridge. The molecular weight of the subunit would then be about 18,000.

A polypeptide with alanine at its C terminus, isolated from human VLDL apolipoprotein, occurs in two polymorphic forms. Brown *et al.* (1970) have reported that the difference in the sialic acid content of the otherwise identical polypeptides could account for the polymorphism. Since we have previously shown (Abraham *et al.*, 1960) that chicken apoVLDL contains galactose, mannose, glucosamine, and sialic acid, it is possible that a polypeptide in chicken apoVLDL could also exist in different polymeric forms.

The presence of a small percentage of polypeptides with molecular weights of 40,000 or more in chicken apoVLDL and chicken apoLDL were shown by disc gel electrophoresis. If these latter polypeptides are different proteins, rather than polymers of the major polypeptide constituent of apoVLDL, we have, thus far, been unable to identify them by immunochemical methods and end-group analysis. The release of both alanine and leucine from chicken apoHDL by hydrazinolysis

suggests the presence of at least two polypeptides. However, the question of heterogeneity of chicken apoHDL remains unresolved primarily because the protein forms aggregates which can not be completely dissociated by urea, guanidine, sodium dodecyl sulfate, Triton X-100, or any of the other dissociating agents tested.

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