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# Characterization of apolipoproteins from chicken plasma

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## ABSTRACT

Although functionally similar, the lipoprotein systems of birds and mammals differ in composition. The major apolipoproteins, apo A-I and apo B, are common to all vertebrates; however apo A-II and apo E, functionally important components of mammalian lipoproteins, are absent from chicken plasma. Chicken apo A-I and apo B have been characterized, and several minor apolipoprotein components have been observed in electrophoretic patterns of chicken lipoproteins. In this study a single density gradient ultracentrifugation was used to isolate and subfractionate chicken lipoproteins into density classes. Isolated lipoproteins were delipidated with hexane–isopropanol (3:2). Apolipoproteins were then solubilized at pH 8.5 in 3 *M* guanidine hydrochloride and chromatographed on a 25 × 0.4 cm $C_4$ reversed-phase column using 0.1% trifluoroacetic acid in a gradient of acetonitrile in water. Molecular weights estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and amino acid compositions were compared with those of apolipoproteins from other species in a search for functional similarities. Similarities in composition between the major chicken apolipoprotein and several human apolipoproteins were observed.

## INTRODUCTION

Plasma lipoprotein particles are the major vehicles for lipid transport in the circulatory systems of vertebrates. Lipoprotein profiles and compositions are important indicators of the nature of lipid metabolism in the organism. The chicken, *Gallus demosticus*, has long been considered a suitable animal model for comparative studies of lipid metabolism[1–4] because in both man and aves liver is the main site of *de novo* fatty acid synthesis. However, differences in lipid biosynthesis and transport between birds and man have been documented[5]. These differences include: porto-microns which transport dietary lipids from the gut directly to the liver[6], and a predominance of high-density lipoproteins (HDL) in the plasma of chickens other than the laying hen[7].

Human serum lipoproteins have been characterized in great detail. The major protein constituents of human HDL, apo A-I and apo A-II, are associated with minor amounts of the apo C proteins (-I, -II and -III), and apo E and apo D, each having

a particular function. Apo B with a molecular weight of about 500 000 dalton is the major protein component of low-density lipoproteins (LDL) and is also found in very-low-density lipoproteins (VLDL) from all species.

In the lower vertebrates, lipoproteins are generally characterized by density class following sequential flotation[8] or density gradient ultracentrifugation[9]. The individual lipoprotein classes are then described in terms of specific lipid moieties and total protein. Either of the commonly used centrifugation methods require at least 48 h of centrifuge time which may allow redistribution of lipoprotein components to occur. Accordingly, more rapid isolation techniques are needed for lipoprotein isolation and analysis.

The present study was undertaken to learn more about the mechanisms of lipid transport and deposition in the chicken where apo A-I and apo B have been characterized. Because counterparts to other human apolipoproteins have not been identified in chicken, it is reasonable to look for proteins which may perform the functions of the minor human proteins. A 4-h centrifugation technique[10], lipid extraction and reversed-phase high-performance liquid chromatography (HPLC) were combined to isolate the water-soluble apolipoproteins from the plasma of broiler chicks. These apolipoproteins were characterized by molecular weight and amino acid composition and compared to apolipoproteins from other species.

## EXPERIMENTAL[a]

### *Materials*

Five week old broiler chicks were maintained on a commercial diet at the U.S. Department of Agriculture Poultry Research Laboratory (Georgetown, DE, U.S.A.). After an overnight fast, blood was collected by heart puncture into tubes containing EDTA (1 mg/ml final concentration) and immediately placed on ice. Plasma was prepared from pooled blood of several chicks by centrifugation at 4°C, 2000 *g* for 10 min. To prevent degradation, sodium azide (0.20%), and reduced glutathione (0.5 μg/ml) were added to the plasma[11]. Buffer containing 10 m*M* Tris, 1.5 *M* NaCl, 1 m*M* EDTA, 0.1% $NaN_3$, 0.5 μg/ml reduced glutathione, and 1 m*M* phenylmethylsulfonyl fluoride at pH 8.5 (density, $d = 1.006$) was used throughout, with modifications as noted.

### *Lipoprotein isolation*

Lipoprotein fractions were isolated from plasma by density gradient ultracentrifugation using the single vertical spin method described by Chung *et al.*[10]. A 10-ml volume of plasma was adjusted to a density of 1.35 g/ml by the addition of 5.94 g KBr and placed in a 40-ml Quickseal polyallomer tube (Beckman, Palo Alto, CA, U.S.A.). A peristaltic pump was used to layer 10 ml of buffer adjusted to 1.20 g/ml by the addition of 0.3265 g/ml KBr over the plasma. The tube was then filled with buffer ($d = 1.006$ g/ml) to a final volume of 40 ml and sealed. Ultracentrifugation was performed at 15°C in a Model L8-70 (Beckman Instruments) preparative ultra-

[a] Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

centrifuge in the slow acceleration mode using the VTi50 vertical rotor for 3.5 h at 50 000 rpm (242 000 *g*). After completion of the centrifugation, a 2-ml fraction containing VLDL was removed from the top of each tube with a Pasteur pipet, the tops were then cut off and the tubes emptied by upward flow fractionation using an HBI-Haake Buchler (Saddle Brook, NJ, U.S.A.) fraction recovery system. Fractions were separated on the basis of color, the refractive index of each fraction was measured, and the density calculated. Whole plasma and lipoprotein fractions were stained for lipid with Sudan Black, and electrophoresed on a discontinuous polyacrylamide-gel gradient (separating gel, 3.6%, spacer gel 2.5%, sample gel 3.3%) constructed in tubes as described by Naito and Wada[12].

*Apolipoprotein preparation*

Apoprotein fractions were prepared by extracting 2 ml of a lipoprotein fraction with 5 ml of hexane–isopropanol (3:2) (v/v) followed by 4 ml of hexane alone[13]. Aqueous layers were dialyzed in 3500 molecular weight cutoff tubing against buffer containing 3 *M* guanidinium hydrochloride (GdnHCl) to reduce the salt level while preventing precipitation of the apoproteins. Apolipoprotein fractions in 3 *M* GdnHCl were concentrated to about 4 mg/ml in an Amicon filter cell with a UM2 membrane (Amicon, Danvers, MA, U.S.A.).

*Reversed-phase HPLC*

A Varian (Sunnyvale, CA, U.S.A.) System 54 liquid chromatograph with a Bio-Rad (Richmond, CA, U.S.A.) Hi-Pore RP-304 reversed-phase column (25 × 0.4 cm I.D.) was operated at 50°C with a flow-rate of 1.2 ml/min. Elution of proteins from the column was monitored at 214 nm with a Varian UV-50 detector. Solvents used were: solvent A, 0.1% trifluoroacetic acid (TFA, Sequanal grade, Pierce, Rockford, IL, U.S.A.) in water; solvent B, 0.1% TFA in acetonitrile (HPLC grade, American Burdick and Jackson, Muskegon, MI, U.S.A.). The composition of the eluent was varied by two linear gradients, initially from 25 to 35% B at 5 min and then to 53% B at 30 min. At 33 min the concentration of B was increased to 75% for 10 min after which the column was reequilibrated to the starting conditions.

*Characterization of proteins*

Electrophoretic patterns of the apolipoprotein fractions were obtained by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) on an 8–25% gradient gel using the PhastGel System (Pharmacia, Piscataway, NJ, U.S.A.). Estimates of protein molecular weights were made by comparison with a standard protein mixture run at the same time. The composition of protein peaks separated by HPLC were determined by amino acid analysis. Fractions with identical elution times were collected from several HPLC runs, pooled and dried under nitrogen, then hydrolyzed in sealed evacuated tubes at 110°C for 24 h with 5.7 *M* HCl, containing 0.05% phenol[14]. Analyses were performed on a Beckman (Fullerton, CA, U.S.A.) 119 CL amino acid analyzer, using the standard 90-min single-column hydrolyzate protocol. Amino acid compositions of peak materials were compared with published compositions of apolipoproteins of other species using the algorithm of Cornish-Bowden[15] for relating proteins by amino acid composition. Compositions of proteins to be compared were obtained from the Protein Identification Resource (National

Biomedical Research Foundation, Washington, DC, U.S.A.), sequences are available for each of these proteins.

## RESULTS AND DISCUSSION

Hermier *et al.*[16] showed that the lipoprotein classes of chicken plasma could be separated by density gradient ultracentrifugation. The single vertical spin technique[10] adapted here proved equally effective. VLDL ($d < 1.016$) formed a thin opalescent film on the top of each centrifuge tube. A layer of clear salt solution separated the VLDL from the LDL ($d = 1.020$–$1.046$) layer, a pale yellow, slightly turbid band. After a second clear, colorless layer of salt solution, the HDL ($d = 1.052$–$1.130$) fraction formed a clear deep yellow band. Separation of lipoprotein fractions after ultracentrifugation was aided by the carotenoid pigments associated with the LDL and HDL layers. The electrophoretic pattern (not shown) obtained for prestained whole plasma and fractions from the density gradient on discontinuous gels in tubes confirmed the separation. Electrophoresis was continued until the HDL fraction reached the bottom of the 3.6% separating gel, at this time the VLDL fraction remained at the interface between the sample gel and the spacer gel (2.5% acrylamide), the LDL fraction had entered the separating gel and moved about 10% of its length.

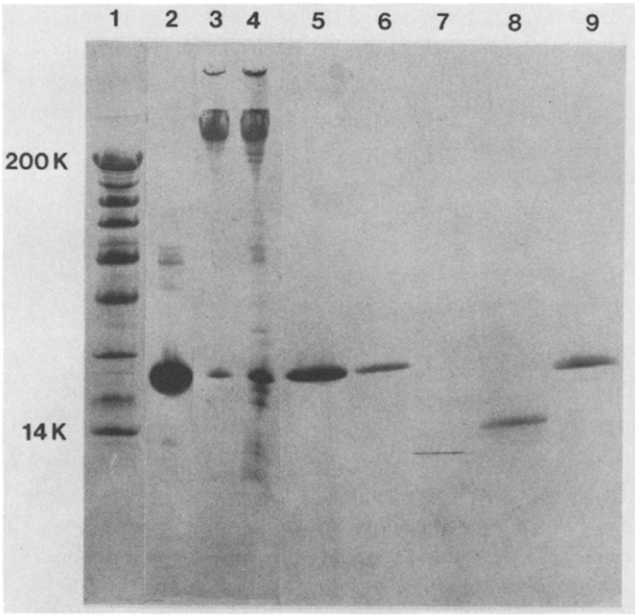


Fig. 1. SDS–PAGE (8–25% gel) of the apolipoproteins from chicken plasma. Molecular weight standards in lane 1 are from the top: myosin, 200 000; $\beta$-galactosidase, 116 250; phosphorylase *b*, 97 400; bovine serum albumin, 66 200; ovalbumin, 43 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 400. Lanes 2–4 are HDL, LDL and VLDL fractions after density gradient ultracentrifugation and delipidation. Lanes 5–9 are RP-HPLC fractions of the apolipoproteins: lane 5 is material from peak 5 (Fig. 2); lane 6 = peak 4; lane 7 = peak 3 (a very faint band with molecular weight of about 11 000 dalton; lane 8 = peak 2; lane 9 = peak 1. K = Kilodalton.

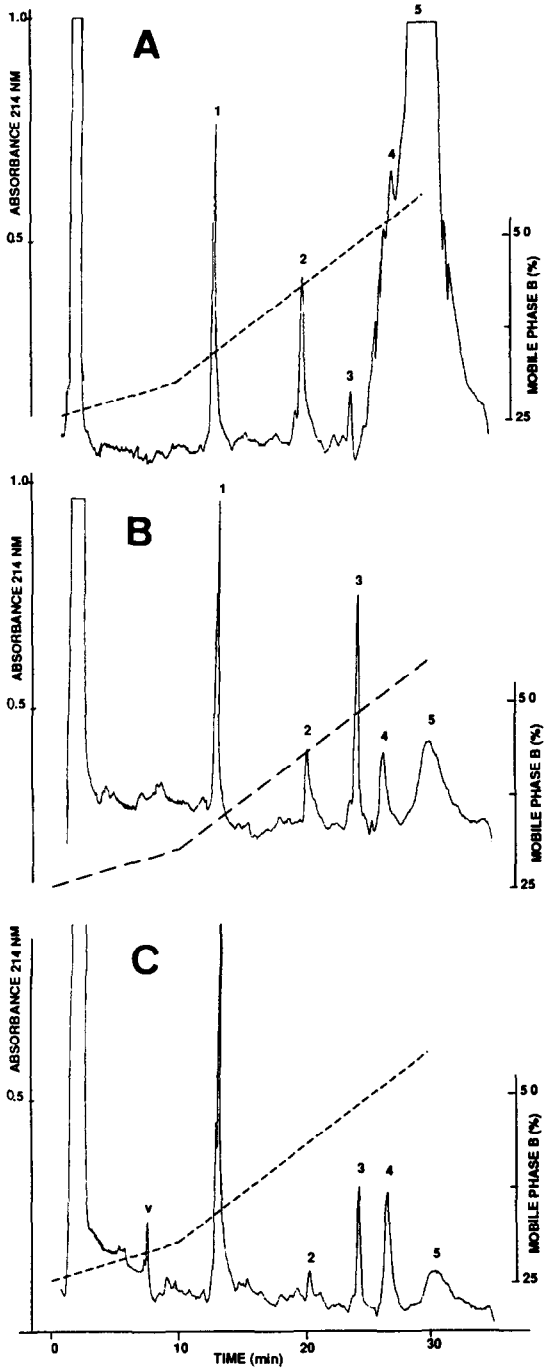


Fig. 2. Separation by RP-HPLC of apolipoproteins from chicken plasma. (A) HDL fraction; (B) LDL fraction; (C) VLDL fraction.

Extraction with lipid solvents removed carotenoid pigments as well, leaving colorless apoproteins in solution. The SDS-PAGE patterns obtained for centrifuged and extracted lipoprotein classes are shown in Fig. 1 (lanes 2–4). The apolipoproteins of chickens, as noted previously[16], are more uniformly distributed throughout the lipoprotein classes than are those of mammals. All fractions had a major band in the 25 000–30 000-dalton molecular weight range; both VLDL and LDL fractions had a smear of proteins or protein fragments with molecular weights greater than 200 000 dalton. HDL fractions had bands between 50 000 and 75 000 dalton which appear to be plasma proteins, not lipoproteins. These bands were more prominent in the electrophoretic patterns of fractions with $d > 1.130$. Several bands with molecular weights lower than 20 000 dalton were also apparent when the gels were heavily loaded.

Fig. 2 shows the HPLC chromatograms of the apolipoproteins from each of the lipoprotein classes. The soluble protein content of HDL and LDL was separated into five peaks eluting between 13 and 29 min. The same five peaks appeared in the chromatogram of VLDL, along with a small additional peak at 8.3 min. Fig. 1 (lanes 5–9) shows the electrophoretic patterns of the peak material from the HPLC experiments. Table I summarizes the molecular weights and relative amounts of the proteins in each class. Apo B and the high-molecular-weight fragments apparent on SDS-PAGE were not sufficiently soluble even in 3 *M* Gdn HCl to be isolated by the HPLC technique.

The amino acid compositions of peak material from each of the five major HPLC bands are given in Table II. Mean residue weights and average hydrophobicities[17] are included to emphasize differences in composition. Larger amino acid residues tend to be more hydrophobic than smaller ones, so it is not surprising that both the mean residue weight and the average hydrophobicity are positively correlated with retention volume on the reversed phase column.

Table III gives $S\Delta n$ values for comparison of the amino acid composition of the protein in each chromatographic peak with lipoproteins of known composition and with each other. The $S\Delta n$ values were calculated with the formula derived by Cornish-Bowden[15]:

TABLE I

DISTRIBUTION OF APOLIPOPROTEINS

| *Peak*[a] | *Elution time (min)*[b] | *Molecular weight*[c] | *Peak area* | | |
|---|---|---|---|---|---|
| | | | *HDL* | *LDL* | *VLDL* |
| v[d] | 8.28 | | | | 0.055 |
| 1 | 13.37 ± 0.11 | 22 300 | 0.021 | 0.072 | 0.228 |
| 2 | 20.06 ± 0.51 | 14 200 | 0.032 | 0.091 | 0.179 |
| 3 | 23.35 ± 0.65 | 11 400 | 0.005 | 0.152 | 0.196 |
| 4 | 25.92 ± 0.36 | 25 500 | 0.132 | 0.150 | 0.172 |
| 5 | 29.00 ± 0.42 | 23 500 | 0.809 | 0.535 | 0.169 |

[a] Peak numbers are referenced to the chromatograms in Fig. 2.
[b] Elution times are the average for HDL, LDL and VLDL with variation.
[c] Molecular weights are estimated from the SDS-PAGE, see Fig. 1.
[d] Insufficient material to obtain molecular weight or amino acid composition for this peak.

TABLE II

AMINO ACID ANALYSIS

| *Amino acid* | *Mole%*[a] | | | | |
|---|---|---|---|---|---|
| | *Peak 1* | *Peak 2* | *Peak 3* | *Peak 4* | *Peak 5* |
| Ala | 5.9 | 6.7 | 8.0 | 8.8 | 8.5 |
| Arg | 6.9 | 4.8 | 2.4 | 7.6 | 7.8 |
| Asp | 9.9 | 7.1 | 8.6 | 7.1 | 7.0 |
| Cys | 0.0 | 0.0 | 0.0 | 1.7 | 0.7 |
| Glu | 11.8 | 17.5 | 14.9 | 20.8 | 20.9 |
| Gly | 15.5 | 14.1 | 9.3 | 2.8 | 1.8 |
| His | 2.6 | 3.9 | 4.7 | 0.4 | 0.3 |
| Ile | 3.1 | 2.2 | 1.8 | 2.2 | 2.0 |
| Leu | 8.2 | 9.0 | 8.1 | 13.9 | 14.4 |
| Lys | 2.4 | 4.2 | 7.5 | 9.4 | 9.9 |
| Met | 0.0 | 0.0 | 0.0 | 0.1 | 1.5 |
| Phe | 4.1 | 3.8 | 4.1 | 2.2 | 2.2 |
| Pro | 5.8 | 5.4 | 0.0 | 5.6 | 6.4 |
| Ser | 8.5 | 10.1 | 11.2 | 4.3 | 4.1 |
| Thr | 7.0 | 6.0 | 8.2 | 4.9 | 4.4 |
| Tyr | 1.1 | 2.1 | 2.3 | 3.0 | 2.9 |
| Val | 7.2 | 3.0 | 7.8 | 5.2 | 5.3 |
| mrw[b] | 104.69 | 106.45 | 108.20 | 114.59 | 115.82 |
| Hb[c] | 1.00 | 0.95 | 0.94 | 1.20 | 1.24 |

[a] Based on the average of three sets of chromatographic experiments.
[b] mrw = the mean residue weight for the protein.
[c] Hb = the average hydrophobicity[17].

$$S\Delta n = 0.5 \sum (n_{iA} - n_{iB})^2$$

where $n_{iA}$ *and* $n_{iB}$ are the mole fraction of residue (i) in protein A or B respectively. $S\Delta n < 0.42$ is a strong indication of relatedness for proteins A and B, while $S\Delta n > 0.93$ suggests no relatedness for the two proteins[15]. The requirement that proteins to be compared must be of equivalent size[15] has been relaxed somewhat in the calculations reported here because it is reasonable to anticipate some degree of relatedness among a single class of proteins.

For all three lipoprotein classes, the protein in peaks 4 and 5 is mostly apo A-I[18]. Multiple HPLC peaks for human apo A-I have been attributed to the presence of multiple oxidation states for methionine and considered to be an artifact of preparation[11]. The compositions of these two peaks correlated very strongly with each other (Table IV) and with apo A-I from chicken[18] ($S\Delta n$ = 0.07 and 0.05), and humans[19] ($S\Delta n$ = 0.15 and 0.17). Peaks 4 and 5 also correlated moderately with human[20] ($S\Delta n$ = 0.47 and 0.52) and *Rhesus macaque*[21] ($S\Delta n$ = 0.58 and 0.62) apo A-II and with human[22] ($S\Delta n$ = 0.57 and 0.63) and rat[23] ($S\Delta n$ = 0.53 and 0.57) apo E. These peaks had a very weak correlation with human apo C-II[24] ($S\Delta n$ = 0.92 and 0.99). In contrast human apo A-II, apo E and apo C-II are not compositionally related ($S\Delta n$ = 1.1 to 1.7). Thus, the functions of these minor HDL apoproteins which are apparently absent in chickens may be incorporated into avian apo A-I.

TABLE III

COMPARISON OF ISOLATED CHICKEN PROTEINS WITH APOLIPOPROTEINS OF OTHER SPECIES

Comparison of the amino acid composition of each HPLC peak from chicken apolipoprotein is with sequenced apolipoproteins. C A-I = chicken apolipoprotein A-I[18]; H A-I = human apo A-I[19]; H A-II = human apo A-II[20]; M A-II = *Rhesus macaque* apo A-II[21]; R A-IV = rat apo A-IV[29]; H A-IV = human apo A-IV[30]; H C-I = human apo C-I[31]; H C-II = human apo C-II[24]; H C-III = human apo C-III[25]; H D = human apo D[32]; H E = human apo E[22]; R E = rat apo E[23]; C Vit = chicken apovitellenin[26].

| *Peak* | *Molecular weight (dalton)* | *SΔn values*[15] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *C A-I, 28 000* | *H A-I, 28 000* | *H A-II, 9000* | *M A-II, 9000* | *R A-IV, 42 000* | *H A-IV, 42 000* | *H C-I, 7000* | *H C-II, 9000* | *H C-III, 9000* | *H D, 21 000* | *H E, 34 000* | *E E, 34 000* | *C Vit 9000* |
| 1 | 22 000 | 1.96 | 1.59 | 1.86 | 2.06 | 1.73 | 1.98 | 2.34 | 1.82 | 1.53 | 1.27 | 2.12 | 1.71 | 1.38 |
| 2 | 14 000 | 1.41 | 1.05 | 1.25 | 1.40 | 4.26 | 4.17 | 1.79 | 1.21 | 1.32 | 1.52 | 1.50 | 1.34 | 1.71 |
| 3 | 11 000 | 1.28 | 0.98 | 0.83 | 0.89 | 3.60 | 3.63 | 1.17 | 0.75 | 0.52 | 1.15 | 1.79 | 1.56 | 1.11 |
| 4 | 25 000 | 0.07 | 0.15 | 0.47 | 0.58 | 4.52 | 4.51 | 1.09 | 0.92 | 1.55 | 1.21 | 0.57 | 0.53 | 1.06 |
| 5 | 23 000 | 0.05 | 0.17 | 0.52 | 0.62 | 4.06 | 4.07 | 1.11 | 0.99 | 1.68 | 1.29 | 0.63 | 0.57 | 1.16 |

TABLE IV

INTERNAL COMPARISON OF PROTEIN COMPOSITIONS

Peak numbers refer to HPLC traces.

| *Peak* | *SΔn values*[15] | | | | |
|---|---|---|---|---|---|
| | *Peak 1, 22 000* | *Peak 2, 14 000* | *Peak 3, 11 000* | *Peak 4, 24 000* | *Peak 5 25 000* |
| 1 | 0 | | | | |
| 2 | 0.37 | | | | |
| 3 | 0.77 | 0.56 | | | |
| 4 | 1.91 | 1.30 | 1.32 | | |
| 5 | 2.14 | 1.51 | 1.54 | 0.03 | 0 |

The composition of peak 3 correlated weakly with apo A-II ($S\Delta n$ = 0.82 and 0.89) and somewhat more strongly with apo C-II ($S\Delta n$ = 0.75) and apo C-III ($S\Delta n$ = 0.52). As these proteins are all in the 9000–12 000 daltons molecular weight range peak 3 protein may represent a real homologue with apo C-III[25], apo C-II, or apo A-II, and should be examined further. Alternatively, this similarity may imply a lipase activator role for peak 3 protein as has been demonstrated for human apo A-II[26] and apo C-II[24]. Proteins in peaks 1 and 2 were not correlated with any mammalian proteins, yet these two are apparently related to each other (Table IV). No other significant correlations with mammalian apolipoproteins were observed.

The poor correlation ($S\Delta n$ = 1.06 to 1.70) between any of the isolated apolipoproteins from broiler chick plasma and apovitellenin[27] is notable because this egg yolk lipoprotein has been isolated from the lipoprotein fraction of plasma from laying hens[28].

By reducing the time during which the various classes of lipoproteins were in contact with each other, the single vertical spin ultracentrifugation employed in this study should minimize the redistribution of components. Nevertheless, except for apo B which was not detected in the HDL fractions, the apolipoproteins were distributed among all density classes. The relative proportions were in general agreement with previous studies of chicken plasma lipoproteins[16]. The HPLC technique, however, gives a rapid profile of the soluble apolipoproteins in each density class and can generate samples for electrophoresis and amino acid analysis more rapidly than traditional separation techniques. The technique could easily be applied to a comparison of apolipoproteins from a variety of species. It also may provide a rapid, sensitive method for following changes in apolipoprotein distribution as affected by disease or dietary changes.

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