

METABOLISM AND NUTRITION

Effects of Phospholipids on the Conformation of Chicken High Density Lipoprotein^{1,2}

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ABSTRACT Apolipoprotein A-I (apo-A-I), the major protein component of chicken high density lipoprotein (HDL), was isolated by hydrophobic interaction chromatography from the lipoprotein fraction of plasma. The apo-A-I was identified from its amino acid composition and molecular weight (by electrophoresis). The isolated protein contained 15% lipid, in the form of neutral lipids and free fatty acids, as compared with 50% lipid, including phospholipid, in intact HDL. The conformation of the isolated protein, as determined from the circular dichroism spectrum, was essentially that of the protein in intact chicken HDL, and similar to that of human HDL. The addition of phospholipid had little effect on the spectrum of this protein. Total delipidation of the protein by extraction with ethanol-diethyl ether mixtures removed the residual lipid, thereby producing an apoprotein with decreased helical structure. The secondary structure of this apoprotein could be restored by the addition of phospholipid. (Key words: high density lipoprotein, hydrophobic interaction chromatography, conformation, lipids)

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INTRODUCTION

The nature of the interactions between lipid and apolipoprotein components of plasma lipoproteins of mammalian species has been studied in detail and reviewed by Morrisett *et al.* (1977). Chicken plasma lipoproteins have been studied primarily as they relate to egg yolk proteins (Burley *et al.*, 1984) or as models for lipoprotein synthesis (Jackson *et al.*, 1976). A major role of lipoproteins is in the transport of lipid from the site of synthesis or absorption to tissues for utilization as an energy source, cellular structural component, or metabolic precursor.

In contrast to human high density lipoprotein (HDL), which contains significant amounts of both apolipoprotein A-I (apo-A-I) and apolipoprotein A-II (apo-A-II), chicken HDL has only one significant protein component, apo-A-I. Kruski and Scanu (1975) isolated apoprotein from rooster HDL by ultracentrifugal flotation and delipidation with ethanol-ether mixtures. Jackson *et al.* (1976)

modified the above procedure to include gel filtration in urea of the delipidated plasma HDL from laying hens, and obtained apo-A-I. Both of these protein preparations were characterized as similar to human apo-A-I except that they contained isoleucine, were associated with higher levels of stearic acid, and appeared to have an α -helical content 20 to 30% greater than that reported for the human protein (Kruski and Scanu, 1974).

The primary structure of chicken apo-A-I has now been determined by amino acid sequencing of overlapping peptides (Yang *et al.*, 1987) confirming the sequence predicted by Byrnes *et al.* (1987) from cloned chicken intestinal cDNA and by Rajavashisth *et al.* (1987) from cloned chicken liver cDNA. These studies show less than 50% sequence homology between human and avian apo-A-I, but well conserved helical hydrophobic moment (Eisenberg *et al.*, 1982) and hydrophobicity profiles (Kyte and Doolittle, 1982). In other recent work, Ross and Carson (1985) isolated apo-A-I from human serum by hydrophobic interaction chromatography without the use of denaturing solvents, an isolation method expected to produce protein in a nearly native conformation.

In the present study, hydrophobic interaction chromatography was used to isolate protein from the lipoprotein fraction of chicken

¹A preliminary report of this work was presented at the 78th meeting of the American Society of Biological Chemists in Philadelphia, PA. June 10, 1987 (Brown *et al.*, 1987).

²References to brand or firm name does not constitute endorsement by the USDA over others of a similar nature not mentioned.

plasma. The conformation of the isolated protein was estimated from the circular dichroism (CD) spectrum, and the effects of added phospholipids on this conformation evaluated.

MATERIALS AND METHODS

Blood was collected by heart puncture from 5-wk-old broiler chicks fed a commercial diet at the USDA Poultry Research Laboratory (Georgetown, DE). An average of 10 mL of blood was drawn from each chick into a syringe containing 1 mg Na₂EDTA as an anticoagulant. The blood was pooled and cells removed by centrifuging at 2,000 × *g* for 10 min. The plasma was frozen and shipped by overnight express. To minimize degradation, 5,5'-dithiobis-2-nitro-benzoic acid (.60 mg/mL), phenyl methyl sulfonyl fluoride (.35 mg/mL), thimerosal (.08 mg/mL), NaN₃ (.13 mg/mL), and additional Na₂EDTA (.37 mg/mL) were added to the plasma as it thawed (Mills *et al.*, 1984).

Phospholipids (dipalmitoyl phosphatidylcholine, DPPC; palmitoyl lysophosphatidylcholine, PLPC; and dipalmitoyl phosphatidylethanolamine, DPPE) were purchased from Sigma (St. Louis, MO) and dilauroyl phosphatidylcholine (DLPC) from Calbiochem (San Diego, CA). Each lipid gave a single spot when chromatographed on silica gel thin layer plates with chloroform-methanol-water (65:25:4) (vol/vol/vol), and all were used without further purification. All other chemicals were of the highest purity commercially obtainable.

Protein Isolation. Thawed plasma was mixed with an equal volume of 4-*M* NaCl and centrifuged at 100,000 × *g* for 24 h at 5 C in a Beckman SW28 rotor (Beckman Instruments, Inc., Fullerton, CA) to float the lipoproteins (Burley *et al.*, 1984). The top 4 mL, containing the lipoprotein fraction, was removed from each 38-mL tube with a capillary pipet, consisting of a .5 × 32-mm plain glass capillary attached, glass to glass, to a Pasteur pipet using a short piece of fine bore vinyl tubing. The salt concentration of the lipoprotein fraction was reduced to .5 *M* NaCl by a 1:5 dilution with TRIS [tris(hydroxymethyl)aminomethane]-saline (.05 *M* TRIS, .1 *M* NaCl, .01% NaN₃, pH = 7.6). The resulting 20-mL sample was pumped onto a phenyl-Sepharose (Pharmacia, Piscataway, NJ) column (1.5 × 26 cm) equilibrated with TRIS-

saline for hydrophobic interaction chromatography (Carson, 1983; Ross and Carson, 1985). The column was washed with TRIS-saline at 20 mL/h to remove the nonadsorbed plasma protein fraction. Apolipoproteins were then eluted with 300 mL of a 0 to 80% (vol/vol) linear gradient of propylene glycol in TRIS-saline. The gradient was applied without pumping to avoid the build up of excessive pressure. The flow rate decreased as the propylene glycol concentration increased. Fractions (2 mL each) were examined spectrophotometrically at 280 nm to detect protein. The propylene glycol content of each fraction was determined by refractometry. Fractions corresponding to a given absorbance peak were pooled, dialyzed against distilled water, and lyophilized.

Characterization of the Isolated Protein. Lyophilized protein fractions were examined electrophoretically on 1.5-mm thick polyacrylamide (10%) gel slabs using a modification (Basch *et al.*, 1985) of the Laemmli (1970) procedure. Sodium dodecyl sulfate (SDS) was present in the electrode buffer but not in the gel. Gels were stained with Coomassie Blue R-250 and molecular weights of protein bands estimated by comparison with a molecular weight standard mixture (Weber *et al.*, 1972). The purity of a protein was estimated by densitometry (Basch *et al.*, 1985). Amino acid analyses were performed in duplicate on samples from two isolations of protein as described previously (Brown and Greenberg, 1984).

Specifically, samples containing 2 mg protein were hydrolyzed in sealed evacuated tubes at 105 C for 24 h with 5.7-*N* HCl containing phenol (.05%), and then analyzed on a Beckman 119 CL amino acid analyzer using the standard 90-min single column protocol. The amount and type of residual lipid in the apoprotein was determined by extracting the isolated protein twice with diethyl ether at 0 C, and three times with a cold (0 C) ethanol-diethyl ether (3:1) (vol/vol) mixture (Osborne, 1986). Extracts were dried and weighed to determine the total residual lipid. Residual carotenoids were determined from an absorption spectrum of the extracted lipids in chloroform using $E_{446\text{ nm}} = 250$ for a 1-mg/mL solution (Broich *et al.*, 1983). Lipid components were separated by thin layer chromatography on Silica G plates developed with hexane-diethyl ether-formic acid (80:20:2)

CONFORMATION OF PROTEIN

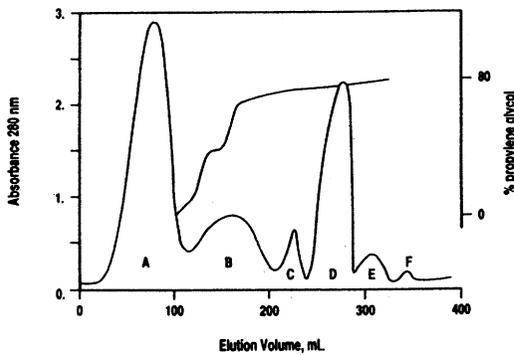


FIGURE 1. Chromatography of the lipoprotein fraction of chicken plasma on phenyl-Sepharose. The profile was obtained using a TRIS (hydroxymethyl)aminomethane-saline wash for the first 100 mL, followed by gradient elution with propylene glycol. Fractions were pooled as indicated (A to F).

(vol/vol/vol) as described by Christie (1982).

Spectrophotometric Measurements. A stock solution was prepared by dissolving 1 mg of protein in 1.0 mL of TRIS-saline buffer (without sodium azide). The solution was filtered through a 13 mm, 4- μ m polycarbonate filter (Nucleopore, Pleasanton, CA) and the concentration estimated from the absorbance at 280 nm using $E = .73$ for a 1 mg/mL sample in a 1-cm pathlength cell calculated as follows (Perkins, 1986): $E_{280 \text{ nm}} = [5550 (\# \text{ TRP}) + 1340 (\# \text{ TYR})]/\text{MW}_{\text{protein}}$. Composition data [2 Trp, 7 Tyr, molecular weight (MW) = 27,967] from the sequence for chicken apo-A-I reported by Yang *et al.* (1987) was used. Stock solutions of soluble lipids (PLPC and DLPC) were prepared by dissolving 1 mg lipid in .5 mL TRIS-saline. Suspensions of DPPC and DPPE were prepared by sonicating 1 mg lipid in .5 mL TRIS-saline, at 50 C under N_2 , in a small culture tube suspended in the cup-horn of a Heat Systems (Plainview, NY) W185 sonifier for 15 min.

To study the effect of added phospholipid on protein conformation, a series of protein-lipid samples was prepared. A 200- μ L aliquot of the stock protein solution was mixed with 10 to 100 μ L of lipid solution or suspension; then the mixture was diluted with TRIS-saline to 1.0 mL, and clarified by centrifugation. The protein concentration in the clarified sample was determined spectrophotometrically after correcting for light-scattering effects as previ-

ously described (Brown *et al.*, 1983). Phospholipid concentrations in the clarified spectroscopic samples were determined by the phosphate method of Ames and Dubin (1960). The CD spectra at 25 C were recorded using a Jasco J41-C spectropolarimeter calibrated with *d*-10-camphorsulfonic acid (Chen and Yang, 1977). Either the protein solution or protein-lipid mixture was placed in a .05-cm pathlength cylindrical cell and scanned from 260 to 190 nm at 10 nm/min, with a time constant of 4 s and a sensitivity of 5.0×10^{-3} deg/cm. The CD spectra were manually digitized, normalized, and analyzed on a Modcomp Classic minicomputer. Ellipticities are reported as $\text{deg cm}^2 \text{ dmol}^{-1}$: $[\Theta]_{\lambda} = (\text{MRW})\Theta_{\lambda}/10bc$, where Θ_{λ} is the observed ellipticity in degrees at wavelength λ , MRW is the mean residue weight (116.5 calculated from the sequence), b is the pathlength in centimeters (.05), and c is the protein concentration in grams per milliliter. The % α -helical structure was estimated from the ellipticity at 222 nm: % α -helix = $([\Theta]_{222 \text{ nm}} + 2,340)(100)/-30,300$ (Chen *et al.*, 1972).

RESULTS

Figure 1 shows the elution profile of the lipoprotein fraction of chicken plasma when chromatographed on phenyl-Sepharose. The lipoprotein fraction applied to the column had a distinct yellow color that stayed at the top of the column during the saline wash, but diffused down the column as the propylene glycol gradient was applied. Although some color was eluted in the fractions comprising Peaks D to F, much remained on the column at the completion of the propylene glycol gradient. The residual color could be removed by washing the column with 2% SDS or by the regeneration of the resin with ethanol and butanol.

Electrophoretic patterns (Figure 2) showed that Peak A, eluted with the TRIS-saline wash, and Peaks B and C, eluted in the early part of the propylene glycol gradient, consisted primarily of serum albumin (MW \approx 66,000) with some variable minor components. Peak D was eluted with 80% propylene glycol and contained a protein (MW \approx 28,000) judged to be more than 90% pure by densitometric analysis. Peaks E and F also contained small amounts of the 28,000 MW protein combined with higher molecular weight proteins. When the protein of

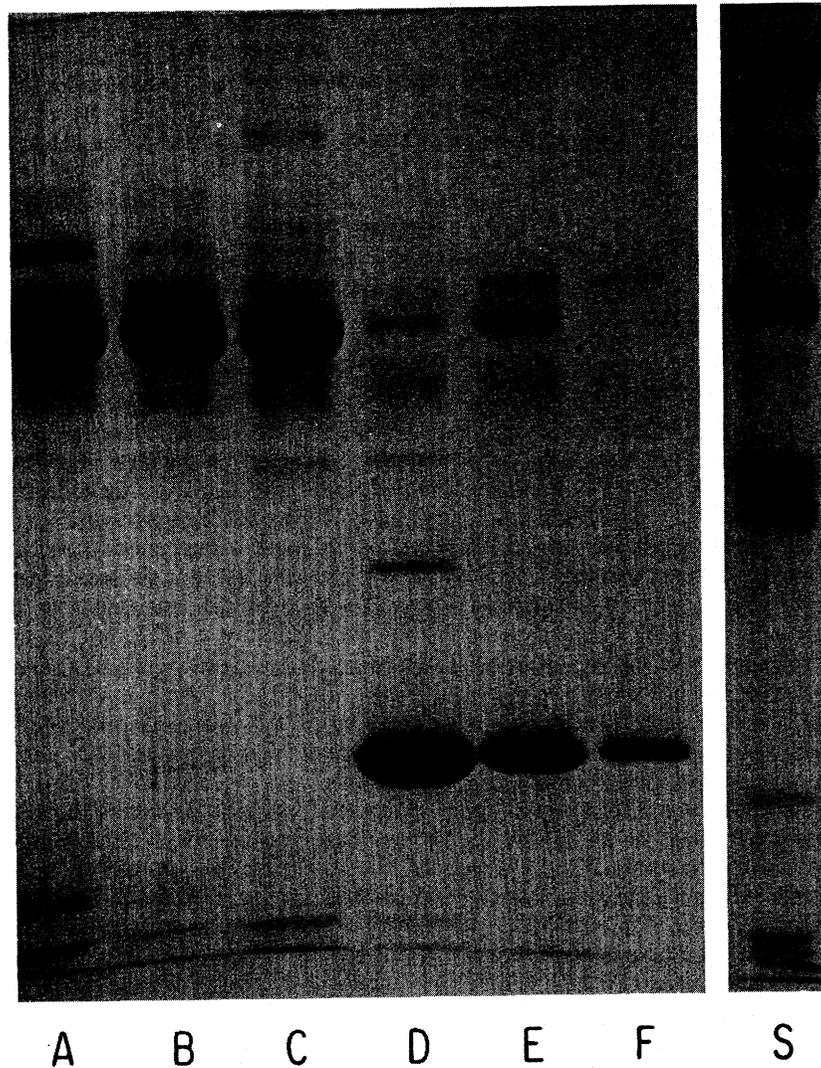


FIGURE 2. Electrophoresis on 10% polyacrylamide gel of proteins in the pooled fractions (A to F) eluted from phenyl-Sepharose. Approximately .05 mg of dialyzed, lyophilized protein in 50- μ L TRIS (hydroxymethyl)aminomethane buffer containing sodium dodecyl sulfate and 2-mercaptoethanol was loaded in each lane. Molecular weight standards in Column S are from the top: myosin, 200,000; β -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.

Peak D was dialyzed and lyophilized, it appeared to be nearly free of lipids except for a slight yellow color. Results of chromatography and electrophoresis of isolated protein from three different plasma samples were identical with 20 to 30 mg of protein obtained in Peak D of each sample.

The amino acid composition of protein from Peak D agreed with that reported in the literature for chicken apo-A-I (Table 1) except

for a second residue of histidine, a 10% excess of proline, and a 5% lower value for lysine. There was no evidence of cysteine or cystine in any of the preparations. These residues would be detected, if present, under the analysis conditions, although not in a quantitative manner.

Ethanol-diethyl ether extraction of 15 mg of isolated protein removed the yellow color as part of a total of 2.2 mg residual lipid. The

CONFORMATION OF PROTEIN

TABLE 1. Amino acid (AA) composition of chicken high density lipoprotein (HDL) prepared by hydrophobic interaction chromatography: comparisons with compositions of chicken apolipoprotein A-I (apo-A-I) and human apo-A-I

Amino acid	Chicken HDL protein ¹			Human apo-A-I ³
	(mol AA/mol P)			
Aspartic acid	19.9 ± .3	(20)	15	16
Asparagine			5	5
Threonine	11.0 ± .4	(11)	11	10
Serine	11.3 ± .9	(11)	11	15
Glutamic acid	48.2 ± .8	(48)	32	30
Glutamine			16	16
Proline	11.2 ± .3	(11)	10	10
Glycine	3.9 ± .8	(4)	4	10
Alanine	21.6 ± .6	(22)	22	19
Half-cystine	ND ⁴	(0)	0	0
Valine	12.2 ± .8	(13)	13	13
Methionine	6.2 ± .3	(6)	6	3
Isoleucine	6.0 ± .1	(6)	6	0
Leucine	32.2 ± .7	(33)	33	37
Tyrosine	7.1 ± .1	(7)	7	7
Phenylalanine	5.6 ± .3	(5)	5	6
Lysine	21.7 ± .6	(22)	23	21
Histidine	2.4 ± .5	(2)	1	5
Arginine	17.6 ± .3	(18)	18	16
Tryptophan	2.5 ± .3	(2)	2	4
Total			240	243
Molecular weight			27,967	28,100

¹Ratios are the average of five analyses, and are presented as (integers) for comparative purposes. Asparagine and glutamine are included under aspartic and glutamic acid, respectively. Tryptophan was determined by the method of Edelhoch (1968).

²From the sequence data of Yang *et al.* (1987).

³From sequence data of Baker *et al.* (1974) and Mahley *et al.* (1984).

⁴ND = Not detected.

visible absorption spectrum (Figure 3) of the extracted lipid contained peaks characteristic of carotenoid pigments, which were estimated from the absorbance at 446 nm to total 15% of the extract. The remaining lipid was shown by thin layer chromatography (Figure 4) to consist of cholesterol, cholesterol esters, and a small amount of free fatty acid (C₁₈ as determined by gas chromatography).

The CD data are summarized in Table 2. The spectrum between 250 and 190 nm of the protein isolated from chicken HDL by hydrophobic interaction chromatography showed a double minimum at 222 and 208 nm, indicative of helical structure. Three separate protein preparations had values of $[\Theta]_{208 \text{ nm}} = -23,086 \pm 800$ and $[\Theta]_{222 \text{ nm}} = -23,061 \pm 1,100$ deg-cm²/dmol, leading to an estimate of 68 ± 3% helix. The CD spectrum of whole chicken HDL fraction was identical to that of the isolated protein at wavelengths between 260

and 210 nm. Photomultiplier saturation and a high noise-to-signal ratio precluded the analysis of data at lower wavelengths. Removal of residual neutral lipids and free fatty acids produced a protein with $[\Theta]_{222 \text{ nm}} = -16,900$ deg-cm²/dmol (48% helix).

When phospholipid in solution or a sonicated suspension was added to the protein, turbidity frequently resulted. To obtain spectrophotometrically useful samples, a clarification step was necessary. In the clarification, both phospholipid and some protein were removed, so that the molar ratio of phospholipid to protein in the CD sample (Table 2, Column 2) represents the soluble complex and residual free components. The constancy of the crossover point (Table 2, Column 3) suggests that the clarification step was adequate for minimizing the concentration of light-scattering particles. Only small changes were observed for $[\Theta]_{208 \text{ nm}}$ and $[\Theta]_{222 \text{ nm}}$ (Table 2,

CONFORMATION OF PROTEIN

TABLE 2. Circular dichroic (CD) parameters¹ of chicken high density lipoproteins (HDL) and its complexes with phospholipids

Protein + lipid ²	[PL]/[P] _{obs}	Crossover nm	[Θ] (deg-cm ² /dmol)		% Helix
			[Θ] _{208 nm}	[Θ] _{222 nm}	
Native HDL ³	17	NO	NO	-22,800	67
HIC ⁴	0	200	-22,300	-22,300	66
+DPPE	2.5	201	-23,100	-22,000	65
+DPPE	4.2	201	-27,100	-24,800	74
HIC	0	200	-22,300	-22,300	66
+DPPC	5.2	201	-23,200	23,300	69
+DPPC	7.5	200	-25,500	-25,500	76
HIC	0	201	-23,900	-24,300	72
+DLPC	2.0	201	-24,800	-24,500	73
+DLPC	4.5	200	-24,000	-23,300	69
HIC + Extracts ⁵	0	201	-15,100	-16,900	48
+PLPC	1.9	202	-17,200	-18,600	54
+PLPC	5.8	202	-20,500	-19,700	58

¹[PL]/[P] is the molar ratio of phospholipid to protein in the CD sample (after centrifuging to clarify), determined from the absorbance spectrum of the protein and phosphate analysis of the solution. The crossover point is the wavelength at which the CD curve crosses the baseline. The mean variation of [Θ]_{222 nm} in three identical experiments was ± 600 deg; for [Θ]_{208 nm} the mean variation was ± 900 deg.

²DPPE = Dipalmitoyl phosphatidylethanolamine; DPPC = dipalmitoyl phosphatidylcholine; DLPC = dilauroyl phosphatidylcholine; PLPC = palmitoyl lysophosphatidylcholine.

³Intact HDL obtained by ultracentrifugation of chicken plasma on a density gradient; NO = not observed.

⁴Protein isolated by hydrophobic interaction chromatography (HIC) contained 15% lipid, but no phospholipid.

⁵Residual lipids extracted with ethanol-ether mixtures.

to the column; the yield of purified apolipoprotein thus increased.

Chicken HDL is composed of nearly equal amounts of protein and lipid (Chapman, 1980). Phospholipids and cholesterol esters comprise the major portion of the HDL lipids of roosters and immature chickens. Phospholipids, which can be electrostatically bound to the protein, might be expected to remain with the protein through hydrophobic chromatography; neutral cholesterol esters should be more effectively removed by interaction with the resin. In practice, phospholipids were preferentially removed, perhaps because the polar head groups were bound to charged residues on the protein surface; this leaves the nonpolar tail available to interact with the hydrophobic resin. In contrast, some neutral lipids may be folded into the protein's tertiary and quaternary structure, which was stabilized by the propylene glycol.

In roosters, nonlaying hens, and immature chickens, 75% of the total lipoproteins are HDL particles (Chapman, 1980). Hydrophobic interaction chromatography proved effective in separating this fraction from the low density

(LDL) and very low density (VLDL) fractions. The LDL and VLDL fractions are characterized by the presence of higher molecular weight apoprotein as seen in Fractions E and F (Figure 2), as well as higher levels of neutral lipids. Hydrophobic interaction chromatography was effective in separating the major protein components of human HDL, apo-A-I, and apo-A-II (Ross and Carson, 1985). Because chicken HDL protein is 93% apo-A-I (Allen, 1987), little further separation was expected. Although the protein preparation by hydrophobic interaction chromatography was not strictly apolipoprotein, lipid depletion was significant (70%).

The CD spectrum of the chromatographically isolated protein was similar to that of whole HDL from chickens. The calculated value of 68% for the helical structure agrees with that determined for human HDL protein (65%) (Kruski and Scanu, 1974). The conformation of the isolated protein was stabilized by the residual nonpolar lipids and stearic acid. In the presence of these residual lipids, the addition of phospholipids had little effect on protein conformation. Removal of residual

lipids from the protein decreased the helical structure to a level equivalent to that of human apo-A-I. The restoration of helical structure when phospholipid was added to this apolipoprotein was equivalent to that observed for the addition of phospholipid to human apo-A-I (Lux *et al.*, 1972; Jonas and Krajnovich, 1977). Earlier reports of significantly greater helical content (90%) for apo-A-I from rooster plasma (Kruski and Scanu, 1975) and from the plasma of laying hens (Jackson *et al.*, 1976) may have been based on the assumption of 4 tryptophan residues as in human apo-A-I (Baker *et al.*, 1974), rather than the 2 tryptophan residues found in chicken apo-A-I (Yang *et al.*, 1987). The general similarities between the two proteins would lead to the assumption of equivalent numbers of chromophoric residues. The consequent underestimation of protein concentration by 35% would result in an overestimation by 35% of helical structure.

In mammals, apo-A-I is synthesized in the liver and intestine, the major sites of lipoprotein synthesis (Smith *et al.*, 1978). The secreted protein is associated only with the high density classes of lipoproteins (Chapman *et al.*, 1981), where it functions in cholesterol metabolism as an activator of lecithin:cholesterol acyltransferase (Soutar *et al.*, 1975) and appears to have a role in the binding of HDL particles to adipocyte plasma membranes (Fong *et al.*, 1987). In contrast, synthesis of chicken apo-A-I has been observed in breast muscle, brain, kidney, and other tissues as well as in the liver and intestine (Blue *et al.*, 1982; Shackelford and Lebherz, 1983). It has been postulated that the apo-A-I synthesized in peripheral tissues of the chicken may play a role in the movement of lipid from those tissues to the liver for degradation (Blue *et al.*, 1982). Although apo-A-I is the only major protein in chicken plasma HDL, it appears in all density classes of the plasma lipoproteins of chicken (Hermier *et al.*, 1985), and may have additional functions similar to those of mammalian apo-A-II and apo-E, which are not a part of the chicken lipoproteins.

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