Isolation and Characterization of a Dog Serum Lipoprotein Having Apolipoprotein A-I as Its Predominant Protein Constituent†

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ABSTRACT: The serum high density lipoproteins (HDL) of normolipemic dogs (beagles) were isolated in the density range of ρ 1.063 to 1.21 g/ml, and characterized in terms of composition and physical properties (flotation and diffusion coefficients, partial specific volume, molecular weight, electrophoretic mobility, ultraviolet absorption, and circular dichroism). The results indicated that canine HDL is a relatively homogeneous class with a molecular weight of about 230 000 and general properties similar to those reported for human HDL. After delipidation, the resulting apolipoprotein, apo-HDL, was fractionated by Sephadex G-200 column chromatography in urea or guanidine hydrochloride solutions. About 90% of the apo-HDL consisted of a protein with a molecular weight of about 28 000, similar in amino acid composition to human apolipoprotein A-I and having the same NH_2 terminus (aspartic acid) and COOH terminus (glutamine) and no carbohydrates. Two other proteins were isolated, one having an apparent mol wt of 55 000 and representing, at least in part, an aggregate of apolipoprotein A-I and the other component with a mol wt of about 8000, not yet characterized. The results indicate that canine HDL, as an intact complex, has general physical properties that lie between those reported for human HDL_2 and HDL_3, and that it differs compositionally from the human products mainly in its predominant content of apo-A-I. These findings together with evidence for the relatively homogeneous nature of the canine HDL provide new prospects for unraveling the relationship between polypeptide composition and HDL structure.

In spite of the relatively extensive use of the dog as an animal model for the study of lipid and lipoprotein metabolism and experimental atherosclerosis (Steiner and Kendall, 1946; Flaherty et al., 1972), including early accounts from this laboratory (Lewis et al., 1952; Robertson et al., 1972; Scanu et al., 1961; Scanu and Szajewski, 1961; Scanu and Page, 1962), relatively little information is available on the physicochemical properties of the circulating lipoproteins of this animal species. Recently, Mahley et al. (Mahley and Weisgraber, 1974; Mahley et al., 1974) compared the lipid composition of the various serum lipoproteins of normal and hyperlipemic foxhounds and mongrel dogs. However, less detailed results were given on the overall structural properties of these lipoproteins and of their apolipoproteins. For the past several months, we have used the dog (beagle) as one of the animal models to complement human studies in our general program which is aimed at elucidating the structure-function relationship in serum lipoproteins. In this report we present a detailed structural characterization of HDL[1] from normolipemic beagle dogs. Besides their intrinsic value, these data provide reference information from which potential alterations induced by dietary and/or chemical agents may readily be assessed.

Materials and Methods

Male beagles, 1–2 years of age, obtained from a single colony, were maintained on a Purina Chow diet and were fasted for about 20 h before bleeding. Blood was obtained from the femoral vein; after clotting, the serum was separated in a Sorvall refrigerated centrifuge at 5000 rpm following the addition of 1 ml of a 5% solution of EDTA (pH 7.0) per dl of serum. The average concentrations of serum cholesterol and triglycerides were 118 and 46 mg/dl, respectively. After separation of the VLDL and LDL by sequential flotation at ρ 1.006 and 1.063 g/ml, respectively, the HDL were isolated in the density range of 1.063–1.21 g/ml at 10 °C, 115 000g by 24-h centrifugation in a Spinco preparative ultracentrifuge. The top fraction of ρ 1.21 g/ml was readjusted to ρ 1.063 g/ml by appropriate dilution, and the specimens were respun for 24 h to remove possible traces of LDL contaminants. The supernatant fluids were brought back to a solution density of 1.21 g/ml by addition of solid NaBr and centrifuged for 48 h at 40 000 rpm, 10 °C, in a Spinco Model L2-65B ultracentrifuge with 40.3 rotors. The purified HDL was dialyzed extensively against 0.15 M NaCl–10^{-3} M EDTA (pH 7.0) and stored at 4 °C for no more than 3 days before use. By agarose electrophoresis (Noble, 1968), the various preparations obtained during the several months of the study gave a single band in the α_1-globulin region. Initially, canine HDL was also examined by CsCl density gradient ultracentrifugation (Scanu et al., 1973) and was found to band as a single component at ρ 1.13 g/ml.

Studies of HDL in the Analytical Ultracentrifuge. The studies were carried out on canine HDL after dialysis against the appropriate buffer. The measurements were generally

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[1] Abbreviations used are: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo-HDL, apoprotein from HDL obtained by delipidation; apo-A-I, major apolipoprotein of apo-HDL.

performed at lipoprotein protein concentrations of <1 mg/ml in a Beckman Spinco Model E equipped with electronic temperature and speed controls and a photometric scanning optical system.

Sedimentation coefficients were determined at 60 000 rpm, 20 °C, at protein concentrations corresponding to 1.0 and 2.0 absorbance units. The midpoint of the absorbance gradient was taken as the position of the protein boundary and was plotted in the usual fashion.

Diffusion measurements were performed with a synthetic boundary cell at 5600 rpm. Since the lipoprotein was less dense than the suspending medium, the boundaries were formed by overlaying of the lipoprotein solution on the buffer instead of the opposite procedure, which is usually employed in the study of higher density proteins. To aid in forming a sharp boundary, the density of the lipoprotein solution was lowered slightly by the addition of 40 μl of water/ml of solution. The superimposed salt gradient resulting from this manipulation did not alter the diffusion of the lipoprotein; this was tested by diffusion measurements with fibrinogen in the ρ 1.21 medium overlaid with a solution containing 40 and 80 μl of water/ml. The values of the diffusion constant (D_{20,H_2O}) for fibrinogen in the two experiments were within 1% of each other and averaged 1.93×10^{-7} cm^2/s, in excellent agreement with the generally accepted figure of 2.0×10^{-7} cm^2/s. Distances (Δr) between 0.25 and 0.75 full absorbance of the protein boundary were taken as measures of spread of that boundary. The validity of this procedure was based on the observation from sedimentation velocity measurements that approximately 95% of the 280-nm absorbing material in the solution migrated as lipoprotein, and that only 5% of the ultraviolet-absorbing material did not migrate. Diffusion coefficients were calculated from the least-squares slopes of plots of $(\Delta r)^2$ vs. time. The adequacy of the experimental procedure was based upon the following: (1) the theoretical zero time (as determined from extrapolation of the plot) agreed within 40 s with the observed zero time at which the boundary began to form, and (2) the observed value of D multiplied by the uncertainty factor (Creeth and Pain, 1967) of the starting time $[t_{0(app)} - t_{0(extrap)}]$ was less than 10^{-4} cm^2. The diffusion measurements were performed at ambient temperature (24 °C) and adjusted to 20 °C by multiplication of the observed diffusion constant by the relative viscosity of the medium at the two temperatures.

Equilibrium centrifugation was performed in accordance with guidelines established by Yphantis (1964) for the meniscus depletion method. An operating speed of 40 000 rpm was selected on the basis of predetermined values of $-s^0_{20,1.21} = 3$ S and an estimated $\bar{v}$ of 0.90, considered to be suitable for analysis at three density values ranging from ρ 1.17 to 1.27 g/ml. We prepared the lowest and highest density solutions by mixing equal volumes of protein in ρ 1.21 medium with either 16 or 35% KBr and measuring the actual densities by pycnometry. Concentrations of protein in the solutions corresponded to an optical density (OD) of 0.4, and scans were performed with the photometer set at an absorbance range of 2.0. The centrifugation time adopted was 24 h, although equilibrium was reached within 18 h. Reduced molecular weights, $M(1 - \bar{v}\rho)$, calculated from the least-square plots of log absorbance vs. r^2 were multiplied by $2 \times 2.303RT/\omega^2$. A plot of the values[2] of $M(1 - \bar{v}\rho)$ thus determined vs. ρ for the three solutions was then extrapolated to determine isopycnic density having $1/\rho_{iso} = \bar{v}$. By using the $\bar{v}$ figures so determined, we calculated the values of M for the three analyses.

The effect of the centrifugal redistribution of salt on the density of the medium was considered as a source of error. The effect was approximated on the basis of the equation of Ifft et al. (1961):

$$\frac{1}{\omega^2 r}\frac{d\rho}{dr} = \frac{M(1 - \bar{v}\rho)}{\nu RT\delta \ln \gamma m/\delta\rho}$$

The activity coefficient (γ) of KBr (Robinson and Stokes, 1955) remains essentially constant over a wide range of molalities (m). Therefore, if one expresses m in terms of percentages (P) of the weight of KBr solutions, the equation could be simplified to express the density gradient ($d\rho/dr$) as a function of P. If $dP/d\rho$ and $\bar{v}$ are determined from the published values in the Handbook of Chemistry and Physics, one can calculate values of the gradient as $d\rho/dr = 2.52 \times 10^{-13}\omega^2 r(1 - 0.34\rho)P(100 - P)$. Redistribution of NaCl in the medium was disregarded as a significant source of error in the density gradient. We calculated that redistribution of 20% KBr in the ρ 1.21 medium, on centrifugation to equilibrium at the 40 000 rpm operating speed, would cause the density at the meniscus of the 0.3-cm fluid column (0.15 cm half-depth) to fall 0.0042 density unit below the initial density of the solution. Since the measurements used for molecular weight determinations were made over only a small (0.04 cm) region at the fluid meniscus, increments in density across the region would be quite small and thus require no correction for the effect due to redistribution of the protein. The correction became important for the calculation of the buoyancy term, $(1 - \bar{v}\rho)$; failure to correct for protein redistribution would lead to estimates which are 1% too low for $\bar{v}$ and 5% too high for M.

Values of $\bar{v}$ were also obtained from density measurements of HDL at various concentrations in the Mettler/Paar precision density meter (Graz, Austria).

Electron microscopy studies of canine HDL by the negative staining technique were conducted as described previously (Pollard et al., 1969).

Studies on the HDL Apoprotein. Apo-HDL was obtained by delipidation of HDL in ethanol–ether as previously described (Scanu and Edelstein, 1971). The dry product was stored at −10 °C under nitrogen. Before use, apo-HDL was dissolved in 0.02 M Tris buffer (pH 8.2) containing 6 M guanidine hydrochloride and 0.1 M dithiothreitol, at 50 °C for 1 h. This pretreatment ensured complete solubilization of the apoprotein. Chromatography was performed in columns (2.5 × 200 cm) of Sephadex G-200 equilibrated in 0.02 M Tris-HCl buffer (pH 8.2) containing either 6 M guanidine hydrochloride or 8 M urea. Separations in 6 M guanidine hydrochloride were achieved at room temperature, whereas those in 8 M urea were run in a constant-temperature room at 6 °C to minimize carbamylation by cyanate (Edelstein et al., 1972). A constant flow of 14 ml/h was maintained. The effluent was continuously dialyzed against 0.005 M NH_4HCO_3 buffer (pH 8.2) with a Biomed dialyzer (Biomed Instruments, Chicago, Ill.), and the elution profile was recorded with an Isco uv monitor (Instrumentation Specialties Co., Lincoln, Nebr.). Fractions of 2 ml were collected in an LKB Model 7000 fraction collector (KLB Produktor, Bromma, Sweden). The appropriate fractions were pooled, immediately lyophilized, and stored at −10 °C under nitrogen.

Immunological Studies. Antibodies to canine HDL and the major apo-HDL protein, apo-A-I, were prepared in male albino New Zealand rabbits. Each animal was given multiple intradermal injections of a total of 100 μg of protein co-soni-

[2] M = molecular weight of the protein; $\bar{v}$ = partial specific volume; ρ = density of the solution; ω = angular velocity; r = distance from the center of rotation; ν = partial molal volume.

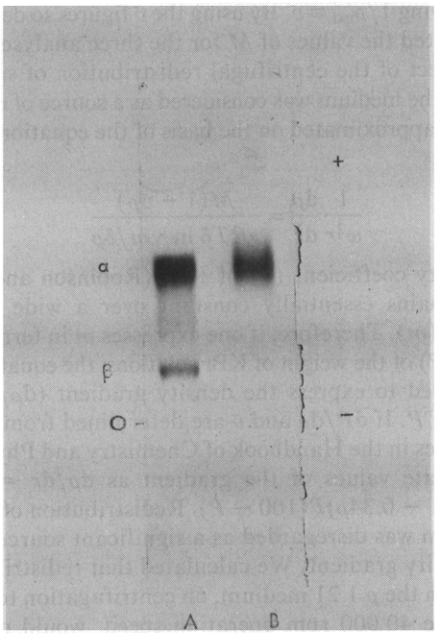

FIGURE 1: Agarose gel electrophoresis of (A) canine serum and (B) canine HDL of ρ 1.063–1.21 g/ml. The conditions were those described by Noble (1968). After electrophoresis, the agarose plates were dried at 60 °C and stained with Fast red 7B. The serum was undiluted (1 μl); HDL contained 7 μg of protein/1 μl.

cated with complete Freund's adjuvant. Six weeks later, the same procedure was followed, except that incomplete Freund's adjuvant was used. The antibodies were concentrated by precipitation of the serum with 2 M $(NH_4)_2SO_4$ (Rapport and Graf, 1967), then resuspended, dialyzed against 0.05 M phosphate buffer (pH 7.0), and stored at −10 °C until use.

Analytical Procedures. Lipid Analysis. For lipid analysis, aliquots of HDL were extracted with chloroform–methanol (3:1, v/v) and washed with 0.05% $CaCl_2$ solution.

Total lipid was measured gravimetrically (Sperry and Brand, 1955). Total cholesterol was determined by a modification of the Abell et al. method (1952), triglycerides by the method of Van Handel and Zilversmit (1957), and phospholipid by the procedure of Fiske and SubbaRow (1925). Ester cholesterol was measured by the digitonin-precipitation technique and assayed by the modified Abell et al. method (1952). For analysis of free fatty acids, the procedure of Falholt et al. (1973) was used. The extracted lipids were also separated by column chromatography (activated Unisil, 100–200 mesh) into neutral and polar lipids. The neutral lipids were further separated on preparative thin-layer chromatographic (TLC) plates (silica gel G, Brinkmann, Westbury, N.Y.) in petroleum ether–diethyl ether–glacial acetic acid (9:10:1, v/v). The cholesterol was methylated and then analyzed on a Hewlett-Packard gas chromatograph, Model 5700 (Hewlett-Packard Co., Chicago, Ill.). The phospholipids were subfractionated on preparative TLC plates (silica gel H) in 0.001 M Na_2CO_3 and developed in chloroform–methanol–water–glacial acetic acid (75:45:6:12 by volume). Each fraction was scraped from the plate and diluted with methanol–chloroform–water (8:1:1, v/v), and quantitated by the Fiske and SubbaRow method (1925) for phosphorus.

Protein Analyses. Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate or 8 M urea (Weber and Osborn, 1969; Davis, 1964). The sodium dodecyl sulfate gels were stained with Coomassie blue according to the procedure of Fairbanks et al. (1971), whereas the 8 M urea gels were stained for 1 h with Coomassie blue by the method described by Weber and Osborn (1969). All gels were destained in 10% acetic acid overnight.

Protein content was determined by the Lowry et al. method (1951), with bovine serum albumin used as a standard. In the case of apolipoprotein A-I, the values were corrected for its amino acid composition. From 280-nm absorbancy readings, the concentration of apo-A-I was also calculated from its extinction coefficient, $E_{280\ nm}^{1\%} = 20.88$.

Amino acid analyses of apo-A-I were performed on a Beckman Model 121 amino acid analyzer. Protein samples were hydrolyzed in a glass-redistilled, constant-boiling HCl (6 N) which contained 0.1% phenol to reduce losses of tyrosine (Moore, 1972). A hydrolysis–time curve was obtained to ascertain destructive losses of serine and threonine. Tryptophan was quantitated by the spectroscopic method of Edelhoch (1967), and by hydrolysis of the protein in 4 N methanesulfonic acid (Moore, 1972). Performic acid oxidations were carried out in separate specimens to determine cysteic acid content.

NH_2-terminal analyses of apo-A-I were done by automated Edman degradation in a Beckman Model 890 sequencer. The released amino acids were detected as their respective phenylthiohydantoins and silylated derivatives by gas–liquid chromatography (Beckman GC Model 45) and thin-layer chromatography (Edelstein et al., 1974).

COOH-terminal analyses of apo-A-I were conducted with the enzymes carboxypeptidases A and Y. Carboxypeptidase A (diisopropyl fluorophosphate treated, Worthington) was repurified as described by Ambler (1967). The digestion was performed in 0.1 M *N*-ethylmorpholine acetate buffer (pH 8.5), with an enzyme-to-protein molar ratio of 1:50, as previously described (Edelstein et al., 1972). Digestion by carboxypeptidase Y (kindly provided by Dr. R. Heinrikson, University of Chicago) was performed in 0.1 M pyridine acetate buffer (pH 5.5), containing 4 M urea. The enzyme-to-protein weight ratio was 1:100 (Edelstein et al., 1974).

Carbohydrate analyses of apo-A-I were carried out according to Dawson (1972). Protein samples were subjected to acid methanolysis (1.0 N HCl in dry methanol at 80 °C for 16 h), trimethylsilylated, and analyzed by gas–liquid chromatography with the use of known standards.

Ultraviolet spectroscopy measurements in the 240–300-nm region were conducted in a Cary Model 14 spectrometer. The protein solutions were thoroughly dialyzed against the appropriate buffers before analysis.

Circular dichroic spectra were recorded in a Cary Model 60S spectropolarimeter equipped with a circular dichroism attachment. The conditions of recording and calculation of molar ellipticity were reported previously (Scanu and Hirz, 1968).

Reagents. All of the chemicals used were reagent grade. Urea (Baker, Chicago, Ill.) was crystallized from 95% ethanol, and the aqueous solutions were further purified through a mixed bed resin (Bio-Rad Ag501-X8, Bio-Rad Laboratories, Richmond, Calif.). High purity guanidine hydrochloride was obtained from Heico, Inc. (Delaware Water Gap, Pa.). Hydrochloric acid, *n*-ethylmorpholine, ethanol, and diethyl ether were freshly distilled.

Results

By agarose electrophoresis, the dog serum was resolved into three lipid stained bands (Figure 1A). The fastest band, migrating in the α_1-globulin region, represented about 65% of the total stainable material by densitometric scanning (Densicord Recording Electrophoresis Densitometer, Photovolt Corp.).

Table I: Summary of the Physical Properties of Canine HDL (Corresponding Values for Human HDL_2 and HDL_3 Are Given for Comparison).

Species	$s^0_{w,20°}$ ($\times 10^{-13}$ cms^{-1})	$D^0_{w,20°}$ ($\times 10^{-7}$ cm^{2}s^{-1})	$\bar{v}$ (cm^3/g)	M^a ($\times 10^5$)	f/f_0	Diameter^b (Å)	10^{-4} deg cm^2 dmol^{-1} $[\theta]_{222}$	$[\theta]_{208}$
Canine HDL	4.01	4.07	0.895	2.30	1.108	90	−2.39	−2.11
Man^c HDL_2	5.45	3.68	0.905	3.60	1.13	100	−2.60	−2.58
HDL_3	4.65	3.93	0.867	1.75	1.31	75	−2.52	−2.50

[a] From sedimentation equilibrium data. [b] From electron microscopy. [c] Data from Scanu et al. (1974).

Of the three bands, one had β mobility (20% of total scanned area) probably representing LDL, and a minor band between the β and α migrating components, comprising about 15% of the total area scanned. The HDL fraction, isolated between ρ 1.063 and 1.21 g/ml, migrated as a single band (Figure 1B) in the area of the α_1 migrating component of dog serum.

Ultracentrifugal Properties of Intact HDL. By flotational analysis at ρ 1.21 g/ml, HDL migrated as a single boundary with the velocity of −2.98 S (at 20 °C). When sedimented to equilibrium, according to the Yphantis method (1964), it formed a single logarithmic concentration gradient as statistically assessed by the method of orthogonal polynomials. The buoyed weight $M(1 - \bar{v}\rho)$, determined from the gradients formed at equilibrium in media of three different densities at high speed (40 000 rpm), proved to be linear with the medium density after correction for redistribution of salts. The same value was obtained from low speed (8800 rpm) equilibrium measurements and densitometric measurements. From such a relationship we determined that canine HDL has a $\bar{v}$ of 0.895 cm^3/g. This value, when entered in the sedimentation equilibrium data, gave a molecular weight for HDL of 2.3×10^5.

Some heterogeneity became apparent on sedimenting HDL to equilibrium at low speed. The values of weight-average molecular weight which were estimated from the least-squares averaged slope over the whole cell and from the slope in the region of the bottom of the cell both agreed with the values obtained at high speed (Figure 2). A steeper slope, possibly due to the presence of a higher molecular weight and seemingly minor component(s), contributed to curvilinearity of the plot in the region of the meniscus in the low speed run. The curvilinearity apparent at low speed may have been obscured by reduced precision of measurements made in this region of the meniscus at high speed. The least-squares averaged slopes determined from low speed equilibrium measurements over the whole cell at three densities extrapolated to essentially the identical isopycnic density determined for the HDL from the high speed run.

The value of the diffusion constant of HDL at ρ 1.21 g/ml, 20 °C, was 4.11 Fick units. By combining the diffusion and sedimentation velocity data at ρ 1.21 g/ml, the molecular weight of canine HDL was 2.13×10^5, a value in excellent agreement with that obtained by sedimentation equilibrium analyses, and also in keeping with the average diameter of 90 Å estimated from electron micrographs of negatively stained canine HDL, which appeared to have a spherical or quasi-spherical shape with no obvious substructure. The frictional coefficient (f/f_0) calculated from the diffusion and velocity sedimentation measurements was equal to 1.108 which indicates that canine HDL is either highly hydrated or asymmetrically shaped with an axial ratio possibly as high as 2.5. At this time we favor the former possibility, in view of the sphericity

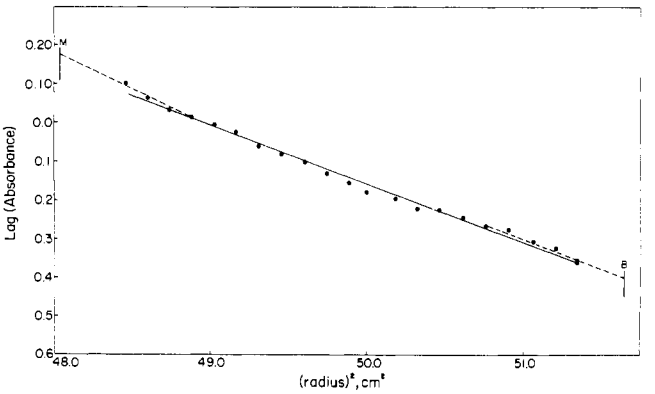

FIGURE 2: Low-speed equilibrium centrifugation of canine HDL. An apparent weight-average molecular weight of 219 000 was calculated for the HDL from (1) the overall linear regression (solid line) of the logarithmic absorbance gradient observed as a function of r^2 after prolonged ($t > 48$ h) centrifugation at 8800 rpm in ρ 1.219 g/ml of medium at a temperature of 23.1 °C, and (2) the co-determined $\bar{v} = 0.895$. A significant but slight nonlinearity was indicated by analysis of data by the method of orthogonal polynomials which yielded a second-order equation $y = 37.3358 - 1.3509x + 0.01202x^2$ as the best fitting relationship between the coordinates, thus warranting calculation of a z-average molecular weight as a quantity differing significantly from the weight-average. Weight-average molecular weights of the protein near the meniscus (M) and bottom (B) of the cell were estimated to be 273 000 and 225 000, respectively, from the least-squares averaged slope of five data points nearest these regions. From the weight-average molecular weights and the concentrations of protein as M, as determined by extrapolation (broken line), the z-average was estimated to be on the order of 290 000. Since the apparent weight-average molecular weight determined from the protein distribution near the bottom of the cell did not differ greatly from that estimated from the overall linear regression it could be inferred that the molecular weight distribution may be skewed in the region of the large sized components.

suggested by the electron microscopic results and particularly by the results of small angle x-ray scattering studies (unpublished observations) showing a great similarity between the canine and human products. The similarity with the human product was also exhibited by the circular dichroic spectra characterized by two minima at 222 and 208 nm with values of molar ellipticity in the range of those previously reported for man. Table I gives a summary of the physical parameters obtained with dog HDL and compares them with reported literature figures for human HDL.

Lipid–Protein Distribution and Lipid Composition. On a weight basis, the ratio of protein to total lipid in canine HDL was in the same range as that reported for human HDL_2 and HDL_3 (Table II). As shown in the table, the percent distribution of esterified and unesterified cholesterol and phospholipids was also similar for the two animal species, except for a lower percentage of glycerides in the dog compared to man. The similarity between canine and human HDL also

Table II: Percent Weight Distribution of Protein–Lipid in Canine HDL.[a]

	Canine HDL	Man[b] HDL$_2$	Man[b] HDL$_3$
Total protein	41.48 ± 2.0	59.0	45.0
Total lipid	58.52 ± 2.0	41.0	55.0
	Lipid Composition (% Lipids)		
Glycerides	0.66 ± 0.06	4.5	4.1
Cholesterol, total	29.32 ± 1.20	21.6	14.6
Cholesterol, esters	24.68 ± 1.20	16.2	11.7
Cholesterol, unesterified	4.64 ± 0.20	5.4	2.9
Phospholipids	27.75 ± 1.30	29.5	22.5
Free fatty acids	1.44 ± 0.14		

[a] The values are the average of four determinations. [b] Values for man were taken from Skipski et al. (1967) and Scanu and Kruski (1975).

Table III: Phospholipid Composition of Canine and Human HDL.[a]

	% of Total Phospholipid by Weight		
		Man[b]	
Phospholipids	Dog	HDL$_2$	HDL$_3$
Phosphatidylcholine	84.3 ± 2	73.8	77.1
Lysophosphatidylcholine	3.4 ± 1	2.0	5.4
Sphingomyelin	6.7 ± 1	14.5	9.2
Phosphatidylinositol	2.3 ± 0.4	2.4	2.4
Phosphatidylethanolamine	3.3 ± 0.5	3.3	2.5

[a] Average of four determinations. [b] Values taken from Skipski et al. (1967) and Scanu and Kruski (1975).

Table IV: Percent Fatty Acid Composition of Cholesterol Esters in Canine and Human HDL.[a]

Ester Fraction	Canine HDL	Human[b] HDL$_2$ + HDL$_3$
14:0	0.52 ± 0.05	
>14:0 < 16:0	0.51 ± 0.12	
16:0	10.69 ± 0.19	10.7
16:1	3.08 ± 0.06	3.2
16:2	0.43 ± 0.03	
18:0	1.03 ± 0.08	1.2
18:1	19.38 ± 0.98	18.9
18:2	56.16 ± 1.04	52.2
20:0	0.25 ± 0.07	
20:3	0.13 ± 0.02	
20:4	7.58 ± 0.46	6.3
24:0	0.25 ± 0.05	

[a] Average of four determinations. [b] Values taken from Skipski et al. (1967) and Scanu and Kruski (1975).

applied to the phospholipid composition (Table III) and to the fatty acid composition of cholesteryl esters (Table IV).

Protein Moiety. Fractionation of Apo-HDL by Column Chromatography. By analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis, apo-HDL was characterized by a major band (Figure 3A) which, based on a calibration plot according to Weber and Osborn (1969), had an apparent molecular weight of 28 000. Four minor bands of mobility slower (mol wt range of 55 000) and faster (apparent mol wt 8000) than the 28 000 dalton component were also present. Polypeptide heterogeneity was also observed in 8 M urea–polyacrylamide gels, which likewise showed the existence of a major and several minor bands. By Sephadex G-200 column chromatography in 8 M urea, apo-HDL was resolved into four peaks (Figure 4), named I, II, III, and IV, according to their order of elution from the column. The same results were obtained in the presence of guanidine·HCl. Peak I generated peaks II and III upon rechromatography, thus representing an aggregate. The amount of peak I varied from time to time, but was definitely increased in aged apo-HDL preparations. This peak will not be considered further in the discussion. Peak II represented, at least in part, an aggregate since upon rechromatography, it partially generated a component eluting as peak III and on sodium dodecyl sulfate–polyacrylamide gel electrophoresis exhibited both the 55 000- and 28 000-dalton components. A detailed analysis of peak II is currently underway. Peak IV was characterized by one major broad, rapidly migrating band on sodium dodecyl sulfate gels and represented less than 3% by weight of the material eluted from the

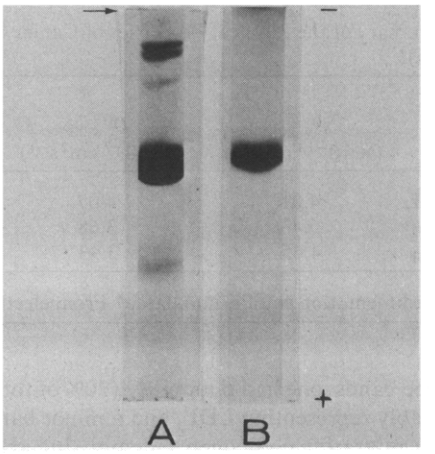

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of (A) canine apo-HDL and (B) canine apo-A-I. Conditions were those described by Weber and Osborn (1969). The gels were stained with Coomassie blue as described by Fairbanks et al. (1971).

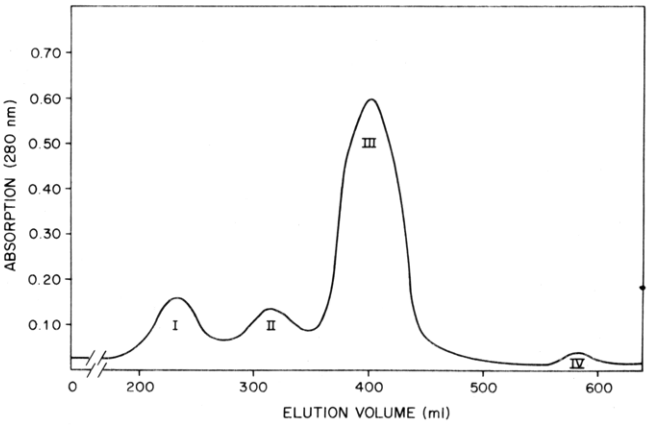

FIGURE 4: Elution profile of canine apo-HDL. Conditions: Sephadex G-200 in 2.5 × 200 cm column; flow rate, 14 ml/h; buffer, 0.02 M Tris-HCl (pH 8.2) containing 8 M urea; amount of protein, 25 mg; t = 6 °C.

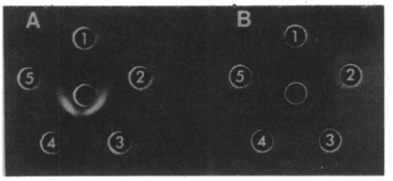

FIGURE 5: Double immunoprecipitin reactions: (A) center well anti-dog HDL; 1, 2, 5, blanks; 3, dog apo-A-I; 4, dog HDL; (B) center well anti-dog apo-A-I; 1, 2, 5, blanks; 3, dog HDL; 4, dog apo-A-I.

Table V: Chemical Composition of Canine Apo-A-I.

Amino Acid	Residues/mol of Protein[b]	
	Canine	Human[a]
Asp[c]	21.82 ± 0.60 (22)[d]	21
Thr[e]	7.32 ± 0.27 (7)	10
Ser[e]	16.64 ± 1.68 (17)	14
Glu[c]	48.12 ± 1.78 (48)	47
Pro	9.77 ± 1.09 (10)	10
Gly	12.97 ± 1.45 (13)	10
Ala	24.00 (24)	19
Val	14.15 ± 1.01 (14)	13
Met	0.96 ± 0.12 (1)	3
Half-Cys	0	0
Ile	2.00 ± 0.15 (2)	0
Leu	35.82 ± 1.56 (36)	39
Tyr	5.96 ± 0.39 (6)	7
Phe	4.12 ± 0.29 (4)	6
Lys	17.34 ± 0.64 (17)	21
His	2.08 ± 0.35 (2)	5
Arg	16.84 ± 0.82 (17)	16
Trp	3.23 ± 0.42 (3)	4
Total residues	243	245
Mol wt[f]	27 411	28 370
COOH terminal	Gln	Gln
NH_2 terminal	Asp	Asp
Carbohydrates	0	0

[a] From Baker et al., 1975. [b] Residues were calculated on the basis of Ala = 24. The values are the mean of ten analyses with their standard deviation. [c] Total value for aspartic and asparagine, and glutamic and glutamine, respectively. [d] Number in parentheses is the nearest integer. [e] Values corrected for hydrolytic losses. [f] From amino acid composition.

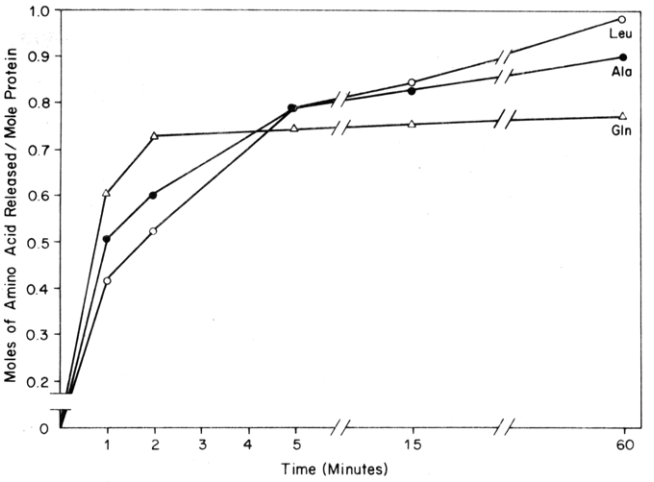

FIGURE 6: Kinetic profile of the release of amino acids by digestion of apo-A-I with carboxypeptidase A. Conditions: 9.4 nmol of protein was digested for the indicated times at 25 °C. The protein to enzyme molar ratio was 50:1. Other conditions were as described in the text.

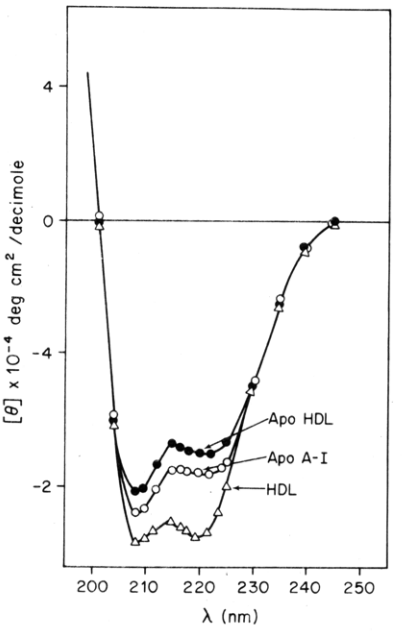

FIGURE 7: Circular dichroic spectra of canine HDL, apo-A-I, and apo-HDL; buffer, 0.01 M $(NH_4)HCO_3$ (pH 8.0).

column. This peak, which was heterogeneous by 8 M urea–polyacrylamide gel electrophoresis and probably comprises members of the A- and C-polypeptides, is currently under investigation. Peak III included about 90% of the apo-HDL eluted from the Sephadex G-200 columns; its properties are therefore described in detail.

Properties of Sephadex Peak III. Peak III gave a single band by both sodium dodecyl sulfate– (Figure 3B) and 8 M urea–polyacrylamide gel electrophoresis. It exhibited a single line of precipitation with antibodies raised in the rabbit against whole dog serum HDL. In turn, antibodies to A-I raised in the rabbit gave a single line of precipitation with whole dog HDL or pure apo-A-I (Figure 5). The amino acid composition of peak III, shown in Table V, indicates a great similarity to human apo-A-I, except for obvious differences noted in the content of methionine (one residue in the dog, three in man), isoleucine (two residues in dog, none in man), and histidine (two residues in dog, five in man). Because of the observed similarities, peak III will be referred to here as apo-A-I.

The molecular weight of canine apo-A-I, estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to Weber and Osborn (1969), was about 28 000; this closely approximates the figure of 27 411 calculated from the amino acid composition (see Table V).

From the kinetics of carboxypeptidase digestion (Figure 6), the COOH-terminal sequence was . . . Leu-Ala-Gln-COOH. By Edman degradation, the partial sequence of the NH_2-terminal fragment was NH_2-Asp-Glu-Pro-Gln-Ser-Pro-Trp-Asp . . . , which is very similar to that reported for human apo-A-I (Baker et al., 1975). Dog apo-A-I, like its human counterpart, had no carbohydrates.

By ultraviolet absorption studies, apo-A-I had an absorption maximum at 280 nm, with a shift toward shorter wavelengths in the presence of guanidine·HCl. Circular dichroic spectra of apo-A-I (Figure 7) exhibited two negative peaks with minima at 222 and 208 nm and a crossover point at 202 nm. The value of the molar ellipticity of apo-A-I at 222 nm was intermediate between those of apo-HDL and HDL.

Discussion

The results of the present studies indicate that serum HDL from normolipemic dogs (beagles) have properties similar although not identical with those of their human counterpart. The similarities are reflected in the fact that the canine HDL float in the density range of high density lipoproteins (i.e., ρ 1.063–1.21 g/ml), have an α_1 electrophoretic mobility, a protein–lipid ratio approximating unity, and a comparable lipid composition. Significant differences were observed, however. First, canine HDL is a rather homogeneous lipoprotein class,[3] with respect to bulk hydrodynamic properties, contrary to human HDL in which two distinct subclasses, HDL_2 and HDL_3, are observed in both the analytical and preparative ultracentrifuges. In consequence, many of the physical parameters, i.e., molecular weight, size, flotation, and sedimentation velocity, of canine HDL lie between those reported for human HDL_2 and HDL_3. It is also significant that the apoprotein of HDL is composed mainly of a 28 000 dalton polypeptide which is similar in amino acid composition to human apolipoprotein A-I and has nearly identical NH_2- and COOH-terminal sequences. This contrasts with human apo-HDL, in which a second major polypeptide, apolipoprotein A-II, is present in dimer form, or with the nonhuman primates where apoliprotein A-II occurs as a monomer. In turn, the canine HDL, by having mostly apolipoprotein A-I and little or no apolipoprotein A-II, resembles the HDL of the cow, pig, chicken, and rat, and probably will be found to be similar to that of other species when a more systematic investigation is carried out (Scanu et al., 1975). The differences between human and canine HDL raise questions of both structural and functional relevance. For example, it would be of interest to establish whether there is a correlation between the observation that HDL is a lipoprotein class relatively more homogeneous than that of man in both density and size, and the observation that it contains mainly one type of protein, apolipoprotein A-I. Another question of interest has to do with the correlation between the structural properties of human and canine apolipoprotein A-I. Present evidence suggests that, in the case of the human product, both apolipoproteins A-I and A-II may be needed in the formation of the HDL complex (Scanu et al., 1975). Since apolipoprotein A-II is not an important component of canine HDL, it follows that, in the dog, apo-A-I is the sole or main contributor to the overall structural organization of these particles. This conclusion encourages an investigation of the primary, secondary, and tertiary structures of this apolipoprotein, its mode of binding, and its affinity for lipids. Furthermore, it has been reported that human apo-A-I is a co-factor in the activity of the LCAT system in vitro (Fielding et al., 1972), and that it enters into the process of steroidogenesis by the adrenal gland (Gwynne et al., 1975). Again, it would be of interest to determine whether these reported activities are exhibited by canine apolipoprotein A-I.

The predominance of apo-A-I in canine HDL has recently been reported by Mahley et al. (Mahley and Weisgraber, 1974; Mahley et al., 1974) and by Nakai and Whayne (1975), the latter in abstract form. In neither study was the exact nature of the minor polypeptides established. In our investigations, fractionation of canine apo-HDL by gel column chromatography led to the detection of protein components with both higher and lower molecular weights than that of apo-A-I. In the case of the higher molecular weight component contained in Sephadex peak II, studies now in progress appear to indicate that, although a large proportion of this peak may represent apo-A-I multimers, there is an additional high molecular weight component chemically distinct from apo-A-I; this component is now being characterized (unpublished). The present results do not allow an assessment as to whether the low molecular weight component(s) in the range of 8000 daltons correspond to any of the A, C, or D apolipoproteins described in man. The resolution of this question must await the isolation of the polypeptides in a pure form, and their characterization.

To sum up, our results have shown that canine serum HDL isolated between ρ 1.063 and 1.21 g/ml is predominantly an apo-A-I lipoprotein. Whereas this observation leaves little doubt about the important structural role of apo-A-I in canine HDL, it leaves unanswered the potential structural participation of the other "minor" polypeptide components, which may or may not correspond to the polypeptides which have been described previously. Studies on this question are in progress in this laboratory.

[3] Mahley and Weisgraber (1974) have presented evidence for an additional high density lipoprotein, HDL_1, which floats in the ultracentrifuge with the low density (ρ 1.006–1.063 g/ml) lipoproteins and which is separable from them by electrophoretic means. We have made similar observations in our dogs and separated HDL_1 from LDL by density gradient ultracentrifugation. In the normolipidemic fasted dog, HDL_1 represented about 10% by weight of the HDL described in this article and its detailed characterization is in progress.

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The Hydrophobic Adsorption of Charged Molecules to Bilayer Membranes: A Test of the Applicability of the Stern Equation†

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ABSTRACT: To describe the hydrophobic adsorption of charged molecules to bilayer membranes, one must recognize that the adsorption produces a change in the electrostatic potential at the surface of the membrane. The surface potential produced by the adsorption of the charged molecules can be described most simply by the Gouy equation from the theory of the diffuse double layer. This potential will tend to lower the concentration of the adsorbing ions in the aqueous phase immediately adjacent to the membrane, a phenomenon which can be described by the Boltzmann relation. The number of adsorbed ions is, in turn, a function of the aqueous concentration of these ions at the membrane solution interface and can be described, in the simplest case, by a Langmuir adsorption isotherm. If the ions are regarded as point charges, the combination of the Gouy, Boltzmann, and Langmuir relations may be considered a simplified Stern equation. To test experimentally the applicability of this equation, one should measure both the charge density and surface potential as a function of the concentration of adsorbing molecules in the bulk aqueous phases. Direct, accurate measurements of one of these parameters, the number of moles of 2,6-toluidinylnaphthalenesulfonate ions bound to vesicles formed from phosphatidylcholine, are available in the literature (Huang, C., and Charlton, J. P. (1972), *Biochemistry 11,* 735). We estimated the change in the surface potential in two independent ways: by means of conductance measurements with "probe" molecules on planar black lipid membranes and by means of electrophoresis measurements on multilaminar unsonicated vesicles. The two estimates agreed with one another and all of the data could be adequately described by the Stern equation, assuming, at 25 °C, a dissociation constant of 2×10^{-4} M and a maximum number of binding sites of $1/70$ Å^2.

A variety of pharmacologically significant molecules are amphipathic in nature and adsorb "hydrophobically" to phospholipid bilayer membranes. The cationic local anesthetics, for example, adsorb to artificial bilayer membranes (Bangham et al., 1965; McLaughlin, 1975) at the same concentration at which they block nerves, but their mechanism of action on the biological membrane is unknown. Anions such as the salicylates enhance the cation and depress the anion conductances of *Navanax* neurons (Barker and Levitan, 1971) and black lipid membranes (McLaughlin, 1973) at identical concentrations, but the mechanism by which these molecules affect the electrical properties of neurons is a matter of debate (Levitan and Barker, 1972; McLaughlin, 1973). Fluorescent

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