2. Part of the enhancement of dimerization when ionic strength is increased can be interpreted as being due to a diminution of long-range nonspecific electrostatic interactions.

3. The major part of the observed enhancement of the dimerization when salt concentration is increased must be attributed to the fact that a certain amount of "structured" water must move from the contact region in order to form the dimer.

4. The structured water is probably of two types: that found in the vicinity of hydrophobic side chains and that found in the vicinity of charged groups in the contact region.

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Hydrodynamic Studies of Human Low Density Lipoproteins. Evaluation of the Diffusion Coefficient and the Preferential Hydration*

Waldo R. Fisher,† Mary Ethel Granade, and Jack L. Mauldin‡

ABSTRACT: This report concerns the evaluation of additional hydrodynamic properties of plasma low density lipoproteins (LDL) isolated from two normal human subjects. Measurements of the diffusion coefficient have been performed in solvents containing 0.2 M KCl or 1.4 M KBr. In both solvents $D^0_{25,w} = 2.17 \times 10^{-7}$ cm²/sec. $\bar{V}$ has been calculated for LDL from *s vs.* density plots in solutions in which the density was adjusted with D_2O or KBr, and the values found were 0.966 and 0.967 ml per g, respectively. The sedimentation coefficient has also been evaluated in D_2O- and KBr-containing solutions, and for these subjects s^0_{25}, ρ 1.20 = −38. The calculated anhydrous molecular weight of LDL from these two subjects is 2.73×10^6 g/mole, and the molecular weight measured in solutions containing KBr does not differ. On the basis of the linearity of the *s vs.* density plot, the identity of the hydrated density of LDL in H_2O–D_2O and in KBr-containing solutions, and the failure of high KBr concentrations to alter the measured value of *D*, it is concluded that LDL is not preferentially hydrated in KBr solutions.

Upon evaluation of the frictional coefficient an f/f_0 ratio of 1.11 is calculated, and, if one assumes these molecules to be spheres, as is suggested by published electron micrographs, then the maximum hydration of LDL is 0.34 g/g of lipoprotein.

The continuing interest in defining the structure of human plasma low density lipoproteins[1] has resulted in a series of reports describing the hydrodynamic properties of these macromolecules (Adams and Schumaker, 1969; Schumaker and Adams, 1969; Del Gatto *et al.*, 1959; Lindgren *et al.*, 1969; Fisher, 1970; Fisher and Mauldin, 1970). In general the molecular weight values reported fall in the range of 2.1–

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[1] Abbreviation used is: LDL, low density lipoprotein.

3.0×10^6 g/mole, and it is probable that the molecular weight of LDL may vary in normal individuals. In most of these experiments the parameters measured have been the sedimentation velocity and hydrated density of LDL, both measured in salt solutions. The calculation of the molecular weight is made by assuming either that the molecule is an anhydrous sphere or by assigning values for the shape and hydration terms. It is further assumed that LDL has insignificant preferential hydration, or salt binding, in the solutions of concentrated salts in which the hydrodynamic properties of lipoproteins are customarily measured. In order to provide an experimental justification for these assumptions the diffusion coefficient and preferential hydration, in KBr solutions, of LDL from two normal subjects have been evaluated. From these measurements the hydrodynamic asphericity and/or hydration of these molecules may be calculated, and any further deviation in the measured molecular weight due to preferential hydration in salt solutions may be determined. The calculation of the "true" hydrodynamic molecular weight of LDL, from these two normal subjects, makes possible the evaluation of the validity of the above-mentioned assumptions, which continue to prove convenient in approximating LDL molecular weights.

In these studies preferential hydration was measured in solutions of KBr since this is a salt commonly used in adjusting the density of lipoprotein solutions for ultracentrifugal studies. In many respects KBr is an ideal solute for this purpose as the relative viscosity of KBr solutions varies minimally from unity over a wide range of KBr concentrations, thus reducing the magnitude of the viscosity correction of the measured sedimentation coefficient (Fisher, 1970).

Methods

All reagents were analytical grade. Glass-distilled water was used throughout. D_2O, 99.8% minimum purity, was purchased from Mallinckrodt. Sephadex G-25 was obtained from Pharmacia.

Aqueous buffers at pH 7.0 were prepared with 0.025 M potassium phosphate containing 100 mg of Na_2EDTA per liter. Solid KBr was then added to adjust the solution to the desired density (Fisher and Mauldin, 1970). D_2O buffer and aqueous buffer containing 0.0125 M potassium phosphate and 100 mg of Na_2EDTA per liter were prepared for the study of the hydrated density of LDL in low ionic strength solvents. Solution densities were measured in triplicate in 2-ml pycnometers at 25°.

LDL was isolated from the fasting blood of two normal subjects (W. F. and J. M.) by a modification of the method of DeLalla and Gofman, using differential-density-flotation centrifugation in KBr solution containing buffered EDTA (Fisher and Mauldin, 1970; DeLalla and Gofman, 1954).

Protein concentrations were determined by a modification of the Lowry method (Bailey, 1967) using bovine serum albumin as a standard. In order to relate the color developed with lipoproteins to that obtained with the albumin standard, LDL samples were dialyzed and lyophilized. Weighed samples were then analyzed by the Lowry method, and the ratio of the specific extinction coefficients of LDL:bovine serum albumin was 0.21. This is consistent with an expected protein content of LDL of 21% (Oncley, 1963) and excludes the problem of lipid interference in color development (Eichberg and Mokrasch, 1969).[2] This factor allows for a direct evaluation of lipoprotein content in LDL solutions.

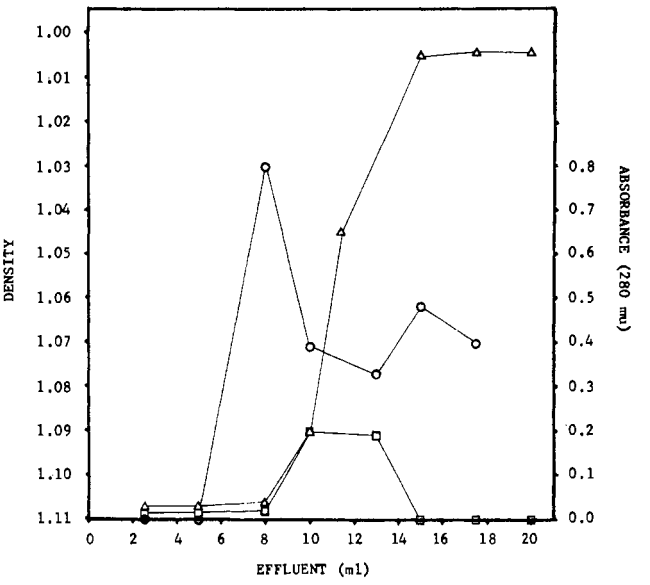

FIGURE 1: Sephadex G-25 chromatographic separation of LDL from aqueous buffer in D_2O-equilibrated column: (○—○) 280-mμ absorbance of fractions following application of LDL dissolved in 1 ml of aqueous buffer; (□—□) density of fractions after application of 1 ml of aqueous buffer followed by D_2O; (△—△) density of fractions after application of aqueous buffer and further elution with the same aqueous buffer. This additional experiment, in which no LDL was applied to the column, was to confirm the elution volume for H_2O.

Viscosity determinations were conducted in an Ostwald single bulb viscometer. Temperature was controlled at 25.0 ± 0.1° by use of a Haake circulating pump. Samples (5 ml) of H_2O, D_2O buffer, and aqueous KBr solutions of 1.06 g/ml and 1.20 g/ml density were studied. Outflow times were reproducible to ±0.03%. Experimentally determined viscosities were used for D_2O solutions. The values for the aqueous solutions were comparable to those in the International Critical Tables (1928); however, values available from these tables were used in the calculations.

D_2O exchange of LDL solutions was carried out by the method of Englander (Englander, 1963). Specifically, two 15 cm × 1 cm columns containing Sephadex G-25 were prepared and equilibrated at room temperature with D_2O containing 0.01 M potassium phosphate.[3]

Concentrated LDL, dialyzed against pH 7.0 phosphate buffer, was brought to room temperature, and 0.5- to 1.0-ml samples, containing approximately 17 mg/ml of LDL, were applied to the column. LDL was eluted in the fraction collected between 6 and 8 ml. This eluted protein was equilibrated at 4° for 24–36 hr in the D_2O eluate. It was then brought to room temperature and applied to a second column and rapidly eluted with buffered D_2O. The protein-containing fractions were combined to yield the deuterium exchanged LDL. Figure 1 demonstrates the separation of the eluted LDL in D_2O from the aqueous buffer in which the protein was applied to the column. The twice-exchanged LDL was ad-

[2] By contrast Adams and Schumaker report an extinction coefficient ratio of 0.26 which is consistent with a previously reported enhancement of color development in LDL (Schumaker and Adams, 1969; Margolis and Langdon, 1966).

[3] Potassium phosphate (0.1 M, pH 7.0) is a minor component contributing 0.025 mole of hydrogen to 111 moles of deuterium in the D_2O solution.

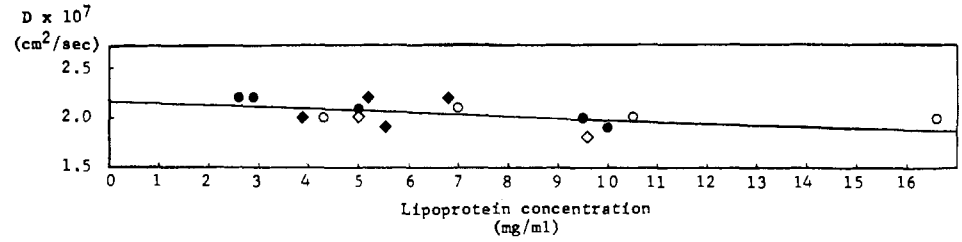

FIGURE 2: Concentration dependence of diffusion coefficient of normal LDL from two subjects ((○) W. F. and (◇) J. M.) at 25°. Measurements made in 0.2 M KCl, solid symbols, or 1.4 M KBr, open symbols.

justed to a volume of 10 ml with buffered D_2O, and the resulting LDL solution was centrifuged in a Spinco Model L-2 preparative ultracentrifuge at 165,000*g* for 22–24 hr in order to concentrate the LDL. Duplicate density determinations on the lower, protein-free fraction of this flotation yielded a solution density of 1.109 g/ml as compared to the density of the original buffered D_2O which was 1.110 g/ml, thus demonstrating that replacement of water by D_2O in this solution was essentially complete.

Sedimentation velocity experiments were performed in a Spinco Model E ultracentrifuge using Schlieren optics. The sedimentation coefficients were determined from plots of log r (the radial distance of the maximum ordinate of the Schlieren peak) *vs.* time and corrected to $T = 25°$, $\rho = 1.20$ (Fisher, 1970).

Hydrated density was evaluated by interpolation of s *vs.* ρ data to $s = 0$ (Fisher, 1970). LDL was studied at four solution densities in aqueous KBr solutions to determine hydrated density in high salt, and in D_2O to determine hydrated density in low ionic strength solutions.

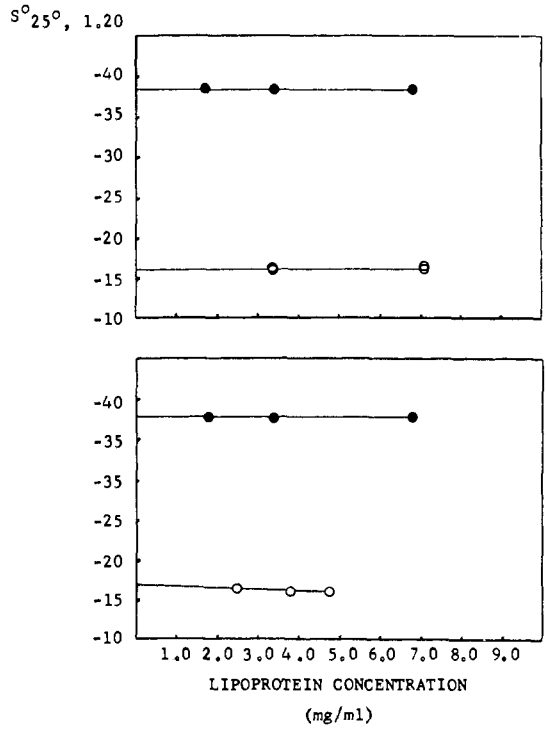

FIGURE 3: Concentration dependence of sedimentation coefficient of normal LDL from two subjects at 25° in ρ 1.20 KBr solutions (●) and D_2O solution (○); top, LDL from W. F.; bottom, LDL from J. M.

The diffusion coefficient was measured in the analytical ultracentrifuge using a synthetic boundary cell. These measurements were performed in solutions of low salt content (0.2 M KCl containing 0.0125 M potassium phosphate buffer) and high salt content (KBr solution of density 1.11 g/ml buffered with 0.025 M potassium phosphate). In the former situation the solvent was layered over the lipoprotein solution, while in the latter experiments a sharper boundary was obtained by layering the lipoprotein solution on top. Diffusion was measured at a centrifugal speed of 5220 rpm and at 25°. Individual diffusion coefficients were calculated using the height–area method at intervals of 15–20 min during the 4- to 5-hr runs, and the data were plotted as a function of the reciprocal of time to minimize the error in the estimation of the zero time of boundary formation. The value, upon extrapolation to the ordinate ($1/T \rightarrow 0$), was the diffusion coefficient at a specific LDL concentration.

Results

The diffusion coefficient of LDL was measured in solutions of low and high salt concentration, 0.2 M KCl and 1.4 M KBr (density 1.11 g/ml). Lipoproteins isolated from both subjects were studied, and the results shown in Figure 2 are from measurements made using two preparations of LDL from one subject and four preparations from the other. On inspection of the data no differences could be seen between the diffusion coefficients of the two subjects or between the values measured in solutions of low or high salt concentration. A line defining the relationship of the diffusion coefficient to LDL concentration was calculated by the method of least squares, and $D^0_{25,w}$ equals 2.17×10^{-7} cm²/sec.[4] The individual points on this plot are derived from measurements of six separate preparations of LDL isolated from two normal subjects, the samples being obtained at varying times over 1 year. The small variation in these measured diffusion coefficients attests to the constancy of the molecular dimensions of LDL in normal subjects.

One previous measurement of the diffusion coefficient of LDL has been reported by Toro-Goyco, who found a $D_{20,w}$ value of 1.85×10^{-7} cm²/sec which may be converted into a $D_{25,w}$ value of 2.11×10^{-7} cm²/sec (Toro-Goyco, 1958). This value was from a single determination at an unspecified LDL concentration.

The sedimentation coefficient of LDL from each subject was measured in aqueous KBr solutions, density 1.20 g/ml. As seen in Figure 3 there is minimal variation with LDL concentration.

[4] In this report a third-place numeral is offset below the line when its statistical significance may be reasonably questioned.

TABLE I: Hydrodynamic Properties of LDL from Two Normal Subjects Measured in Various Solvents.

	High Salt Solution (KBr ρ 1.20)		Low Salt Solutions			
			Aqueous		D_2O	
Salt concentration	2.5 M KBr 0.025 M [$NaPO_4$].		0.0125 M [$NaPO_4$]		0.0125 M [$NaPO_4$]	
ρ solution	1.200		1.002		1.110	
η rel	0.960[a]		1.006[a]		1.238	
Subject	W. F.	J. M.	W. F.	J. M.	W. F.	J. M.
$s^0_{25} \times 10^{13}$	−38.5[b]	−38.0[b]			−16.1	−16.9
Hydrated density	1.034	1.034	1.035[c]	1.035[c]	1.0385[c]	1.0385[c]
$\bar{V}$	0.967	0.967	0.966	0.966	0.963	0.963
$D^0_{25,w} \times 10^7$ cm²/sec	2.17[d]	2.17[d]	2.17[e]	2.17[e]		
$s_{25} \times 10^{13}$ (LDL concentration 3.4 mg/ml)			7.54	7.55	−16.07	−16.08
Molecular weight	2.72×10^6				2.73×10^6	

[a] From International Critical Tables (1928). [b] s^0_{25} ρ 1.20 is the sedimentation coefficient at infinite dilution measured in a solvent of density 1.20 g/ml at 25°. The value is corrected for the relative viscosity of the solvent (Fisher, 1970). [c] Calculated values. Please see text. [d] The solvent for these measurements was KBr of ρ 1.110 (1.4 M) buffered with phosphate. [e] The solvent for these measurements was 0.2 M KCl buffered with 0.0125 M phosphate.

TABLE II: Weight Per Cent Exchangeable Hydrogens in LDL.[a]

Fraction	Grams of Exchangeable H per 100 g of Component	Grams of Component per 100 g of LPL	Grams of Exchangeable H per 100 g of LPL for Each Component	Reference
Peptide[b]	1.30	23	0.30	Margolis and Langdon, 1966
Cholesterol	0.26	8.01	0.02	Skipski *et al.*, 1967
Phosphatidyl-ethanolamine	0.42	0.45	0.002	Skipski *et al.*, 1967
Phosphatidylserine	0.7	0.17	0.001	Skipski *et al.*, 1967
Sphingomyelin	0.27	5.4	0.015	Skipski *et al.*, 1967
Lecithin cholesterol esters triglycerides	0	63	0	Skipski *et al.*, 1967
Total		100	0.34	

[a] Calculations are based on pH 7 for the solution. [b] Assuming two polypeptide chains per 10^5 g of apoprotein.

In like manner the sedimentation coefficient of deuterium exchanged LDL in D_2O (density 1.110 g/ml) was measured and is plotted in the same figure. The results of these studies are tabulated in Table I. In order to correct these measured sedimentation coefficients for the solvent viscosity, η_{rel} of the D_2O buffer was measured and found to be 1.238. This value is consistent with the value for the relative viscosity of pure D_2O at 25° of 1.231 reported by Hardy and Cottington (Hardy and Cottington, 1949).

The hydrated density of LDL in KBr solutions was determined from *s vs.* ρ plots for both subjects (Figure 4), and the partial specific volume of LDL in KBr solution was calculated from the reciprocal of this value. These values are recorded in Table I.

In order to evaluate the hydrated density of LDL in the absence of high salt concentrations, the sedimentation coefficient of the "exchanged" LDL in D_2O buffer of density 1.110 g/ml and the sedimentation coefficient of the LDL studied in 1.002 g/ml of aqueous buffer were determined. Both buffers contained 0.0125 M phosphate and 100 mg of EDTA per l. These values, corrected for the relative viscosity of the solutions, are also recorded in Table I and may be used to calculate the hydrated density of LDL in an aqueous solvent of low salt concentration. In these experiments determining the hydrated density of LDL the lipoprotein concentration was maintained constant at 3.4 mg/ml.

Since these studies were performed in D_2O with deuterium-exchanged LDL, it is necessary to correct the measured sedi-

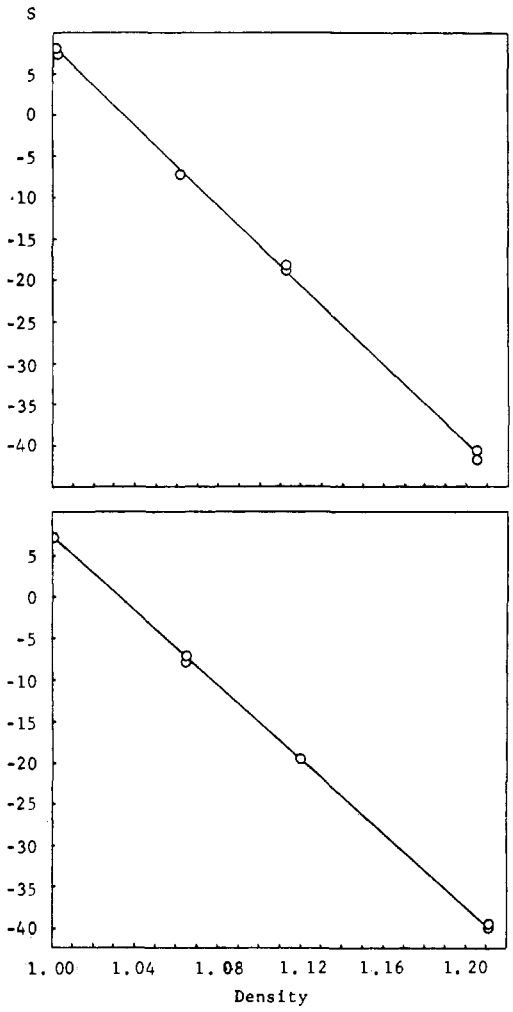

FIGURE 4: *s vs.* density plots for normal LDL in buffered KBr solutions. Lipoprotein concentration 3.4 mg/ml; top, LDL from W. F.; bottom, LDL from J. M. *s* has been corrected for the relative viscosity of the solution.

mentation coefficient of the deuterated lipoprotein for the added mass due to the presence of the deuterium. A calculation of the weight per cent exchangeable hydrogens in LDL was based on published compositional analyses of the molecule tabulated in Table II (Margolis and Langdon, 1966; Skipski *et al.*, 1967). Analysis of this information yields a specific weight increment due to deuterium exchange of 0.0034 g/g of LDL.

In order to evaluate the hydrated density of LDL in a solution of low ionic strength the following equation was developed (please see Addendum for the derivation of this equation).

$$\text{Hydrated density of LDL } (\rho_H) = \frac{\rho_{H_2O}\left(\frac{s_D}{s_H}\right) - \rho_{D_2O}}{\frac{s_D}{s_H} - 1 - X}$$

ρ_{H_2O} and ρ_{D_2O} equal the densities of aqueous and deuterated buffers, s_H and s_D are the sedimentation coefficients of protonated and deuterated LDL, and X is the specific weight increment of LDL resulting from deuterium exchange (0.0034 g/g of LDL). Upon substituting the values from Tables I and II in this equation, the hydrated density of LDL, determined in the presence of low salt concentrations, may be calculated. The average value for the hydrated density of LDL is 1.035 g/ml, which is essentially the same as the value of 1.034 g/ml determined in a solution of high KBr concentration.

On the basis of the data in Table I, one may calculate the molecular weight of LDL utilizing the Svedberg equation:[5] $M = RTs/D(1 - \bar{V}\rho)$. When the molecular weight is calculated using the data measured in solutions of high KBr concentration, the value is 2.72×10^6 g/mole.

In calculating the molecular weight of this lipoprotein in a solvent of low salt content, the sedimentation coefficient measured in D_2O may be used; however, it is then necessary to utilize a value of $\bar{V}$ for the deuterated lipoprotein. This value is readily derived from the hydrated density of LDL in low salt solutions by the relationship $\rho_D = \rho_H(1 + X)$, where ρ_D and ρ_H are the hydrated densities of deuterated and protonated LDL, and X, again, is the specific weight increment resulting from deuterium exchange of the lipoprotein. The resulting value is 0.963 ml/g. The molecular weight of the deuterated lipoprotein is 2.74×10^6 g/mole.[6] One must then subtract the weight of the substituted deuterium (0.0034 g/g of LDL), and the molecular weight of LDL measured in a solvent of low salt concentration is 2.73×10^6 g/mole. Within the limits of our experimental methods the molecular weights of LDL measured in solvents of high KBr and low salt content are indistinguishable. Accordingly, an average molecular weight of 2.73×10^6 g/mole will be assumed for LDL isolated from these two normal subjects.

Discussion

In these studies the partial specific volume of LDL was determined from the reciprocal of the hydrated density as was measured in water and D_2O at low ionic strength. Katz and Schachman have pointed out that $\bar{V}$ measured under these conditions should be identical with $\bar{V}$ determined by pycnometry and in either case should represent the partial specific volume of the dry protein (Katz and Schachman, 1955). Cox and Schumaker documented that for both bovine serum albumin and ribonuclease measurements of $\bar{V}$ by sedimentation in D_2O and H_2O and by pycnometry gave the same results (Cox and Schumaker, 1961a,b), and similar observations have been reported for bovine serum albumin, sodium alginate, and polyvinyl alcohol (Martin *et al.*, 1956). All of these authors, as well as Gagen, have emphasized that values of $\bar{V}$ measured by sedimentation can only be assumed to equal $\bar{V}$ as measured by pycnometry when the ultracentrifugational studies are performed in a two-component system in which selective salt binding or preferential hydration of the macromolecule cannot occur (Gagen, 1966). In order to determine $\bar{V}$ by ultracentrifugation, sedimentation measurements must be performed in solvents of two different densities, and to obtain these data solutions of the macromolecule in H_2O and D_2O are utilized. A measurement of $\bar{V}$ for LDL using pycnometry was reported by Toro-Goyco and the average value

[5] M = molecular weight, R = molar gas constant, T = absolute temperature (degrees Kelvin), s = sedimentation coefficient, D = diffusion coefficient, $\bar{V}$ = partial specific volume of LDL, and ρ = density of the solution.

[6] The diffusion coefficient measured in 0.2 M KCl was used in this calculation. Deuteration of the protein should not alter the value of D.

found was 0.967 ml/g (Toro-Goyco, 1958).[7] This value is comparable to $\bar{V}$ measured by sedimentation in D_2O and H_2O in the present study where a value of 0.966 ml/g was determined.

The molecular weight of a macromolecule, when measured by sedimentation–diffusion, is a function of the anhydrous molecular weight of the substance plus the weight of any preferentially bound water or solute, *i.e.*, associated with the macromolecule in a concentration which differs from that in the bulk solution (Katz and Schachman, 1955). When hydrodynamic measurements are performed in a two-component system, in which no salt is present, the preferential hydration term disappears, and the resultant measured molecular weight is that of the anhydrous particle.[8]

The molecular weight of LDL isolated in these studies was measured in solvents containing only minimal quantities of salt to maintain lipoprotein solubility. The anhydrous molecular weight of these LDL preparations is 2.73×10^6 g/mole. This value is comparable to that reported by Bjorklund and Katz in which their light-scattering measurements on LDL yielded a value of 2.77×10^6 g/mole (Bjorklund and Katz, 1956).[9] Their measurement should also evaluate the anhydrous molecular weight of LDL, and the agreement between the results of two entirely different methods is reassuring.

From the anhydrous molecular weight one may calculate the radius of an anhydrous sphere having the same molecular weight and density of LDL using the relationship $r = (3\bar{V}M/4N\pi)^{1/3}$ (Tanford, 1961). The calculated value is 101 Å, *i.e.*, a diameter of 202 Å. Recent electron microscopic studies of LDL from normal subjects have shown spherical appearing particles with diameters of 216 Å and 216–220 Å (Forte *et al.*, 1968; Gotto *et al.*, 1968). The agreement in the diameter measured by these totally different methods is within 10%.

The evaluation of the diffusion coefficient of LDL makes possible the evaluation of the frictional coefficient. According to hydrodynamic theory

$$f = 6\pi\eta \left(\frac{f}{f_0}\right)_s \left[\frac{3M(\bar{V} - \delta_w \bar{V}_w)}{4N}\right]^{1/3}$$

(Tanford, 1961); f is thus a function of the anhydrous molecular weight and partial specific volume, of the hydration (δ_w) of the particle, and of the deviation of the shape of the particle from a sphere $(f/f_0)_s$. For LDL the last two parameters are unknown; however, if one assumes, on the basis of the electron microscopic data, that LDL is a spherical particle then the maximum possible hydration for LDL in solutions of low salt content is 0.34 g/g of LDL.

Recently there has been increasing interest in the study of lipoproteins using sedimentation–velocity measurements in solutions of high salt content (Schumaker and Adams, 1969; Del Gatto *et al.*, 1959; Lindgren *et al.*, 1969; Fisher, 1970; Fisher and Mauldin, 1970). It has also been recognized that in solutions of high salt content significant preferential hydration of macromolecules occurs and that, unlike simple hydration, preferential hydration alters the measured molecular weight by producing a change in the partial specific volume of the hydrodynamic unit (Katz and Schachman, 1955; Cox and Schumaker, 1961a,b; Hill and Cox, 1965).[10] Because of the need to measure accurately the molecular weight of LDL, it seemed highly pertinent to assess the amount of preferential hydration. This phenomenon was investigated in solutions of KBr since this salt is commonly used to adjust the density of lipoprotein solutions for ultracentrifugational studies.

If one examines the relationship between s and the solvent density (ρ) as defined in the Svedberg equation $s = M(1 - \bar{V}\rho)/Nf$, it is apparent that a plot of s *vs.* ρ should be linear providing the solvent has "no effect on the size, shape, or density of the particles . . . , and that the intercept at $s = 0$ is dependent only on the density of the particles" (Schachman and Lauffer, 1949). This relationship has been investigated for a number of proteins, and both linear and nonlinear dependency has been observed (Schachman and Lauffer, 1949; Katz and Schachman, 1955; Cox and Schumaker, 1961a,b; Hill and Cox, 1965). The data in Figure 4 demonstrate that a linear relationship holds for LDL; however, this does not necessarily indicate a lack of preferential hydration but is also consistent with a constant amount of preferential hydration, as is found under certain conditions for tobacco mosaic virus, ribonuclease, and ovalbumin (Schachman and Lauffer, 1949; Cox and Schumaker, 1961a,b; Hill and Cox, 1965). It then becomes necessary to evaluate the intercept at $s = 0$ both in the presence and absence of salt to determine whether there is a change in the anhydrous density of the protein resulting from preferential hydration. The results of these measurements, recorded in Table I, document that the hydrated density of LDL is the same either in an aqueous KBr solvent or in an H_2O–D_2O solvent.

These data suggest that LDL does not undergo preferential hydration in KBr solutions; however, because the anhydrous density of LDL (1.035 g/ml) and the density of water are so nearly the same, it seemed possible that LDL might be preferentially hydrated, but that the resultant density change would be too small to measure. By contrast, preferential hydration of a protein with an anhydrous density in excess of 1.25 g/ml is readily manifest by a change in apparent hydrated density. Since preferential hydration of a macromolecule alters the frictional coefficient by the addition of an extra hydration term (Katz and Schachman, 1955; Schachman, 1959), an effort was made to demonstrate this phenomenon by measuring the diffusion coefficient in the presence and absence of a KBr-containing solvent. As shown in Figure 2, no difference in D could be demonstrated.

These studies have thus failed to provide evidence for the preferential hydration of LDL in concentrated KBr solvents as evidenced by the linearity of the s *vs.* ρ plot, the identical hydrated density of the lipoprotein in solvents of low ionic

[7] This value was for Fraction 3B, found by Toro-Goyco to be the major LDL-containing fraction from plasma. This lipoprotein had sedimentation and diffusion coefficients similar to those measured for the lipoproteins in this study.

[8] The solvation of a macromolecule, *i.e.*, its association with solvent of the *same* composition as in the bulk solution, is reflected in the frictional coefficient; however, this term is eliminated when the molecular weight is calculated from the experimental parameters, s, D, and $\bar{V}$ (Schachman, 1959).

[9] This molecular weight is for their *sf* 5.9 preparation. The LDL in the present study has an $\eta s_{1.063}$ value of −7 which is converted into an *sf* value of 6 by correcting for the relative viscosity of an NaCl solution of density 1.063 g/ml (Fisher, 1970).

[10] Preferential hydration also increases the magnitude of f, reflecting an increase in the hydration term. When the molecular weight is determined from measurements of s, D, and $\bar{V}$, this effect on f is eliminated; however, in estimates of the molecular weight based only on measurements of s and $\bar{V}$ and performed in salt solutions, the resultant calculated molecular weight would also reflect the deviation of f from the assumed value of f used in the calculation. In either method of determination the change in $\bar{V}$ of the preferentially hydrated particle will be reflected in the molecular weight because of a change in the effective mass of the particle, $M(1 - \bar{V}\rho)$.

strength and in concentrated KBr-containing solutions, and, finally, the failure of the presence of high salt concentrations to alter the frictional coefficient of LDL.[11]

In 1958 Toro-Goyco reported a value for the "water of hydration" of LDL of 0.1 g/g of lipoprotein (Toro-Goyco, 1958). His value was calculated from the partial specific volume of the anhydrous lipoprotein measured pycnometrically (his value of $\bar{V}$ was the same as that measured in the present study, as previously noted) and from the partial specific volume of the "hydrated protein" measured ultracentrifugally in NaCl solutions. This latter value of 0.970 ml/g differs from the value 0.967 ml/g found in the present study. From these data and his calculation it seems evident that Toro-Goyco is reporting preferential hydration in NaCl solutions.

The studies of preferential hydration in KBr- and NaCl-containing solutions indicate a discrepancy in the behavior of LDL, for no preferential hydration is evident in solutions with KBr as solute while Toro-Goyco reports a small but presumably significant amount when NaCl is the solute. Variations in the amount of preferential hydration of specific proteins in solvents with differing univalent salts has been previously demonstrated (Cox and Schumaker, 1961a,b; Hill and Cox, 1965). In the small series of reported studies on the occurrence of preferential hydration of proteins this phenomenon has been uniformly observed (Katz and Schachman, 1955; Cox and Schumaker, 1961a,b; Hill and Cox, 1965; Schachman and Lauffer, 1949; Ifft and Vinograd, 1966). In general, the amount of this hydration is in excess of 0.2 g/g of protein. The apparent failure of LDL to undergo preferential hydration with KBr as a solute, and the low hydration value with NaCl, sets this protein apart from other water-soluble proteins with respect to this physical parameter. Pragmatically, however, these findings are fortunate as they remove the uncertainty of a possible significant error in molecular weight estimations when LDL is studied in solvents of high KBr concentration.

From these studies it is not possible to measure precisely the amount of simple hydration of LDL by the bulk solvent; however, a maximum value has been defined, as previously indicated, from the measured frictional coefficient, and this value of 0.34 g/g is consistent with measurements for other proteins (Tanford, 1961).

Of more practical concern has been the determination of the ratio of the measured frictional coefficient to the hypothetical coefficient calculated for an anhydrous sphere of similar molecular weight. This value of f/f_0 is 1.11. As previously mentioned, it is common practice to estimate lipoprotein molecular weights from measured values of s and $\bar{V}$ and assume that the molecule is an anhydrous sphere. When such calculations are made utilizing the values reported in this study, an estimated molecular weight of 2.33×10^6 g/mole is determined.[12] This value may be corrected to the true molecular weight of 2.73×10^6 g/mole by utilizing the measured frictional ratio, and the difference between these values arises from simple hydration and/or asphericity of the lipoprotein molecule.

[11] In addition, the same molecular weight is calculated using either the values of s, D, and $\bar{V}$ measured in D_2O and H_2O or measured in KBr solutions. Since each of these values would presumably be affected by preferential hydration or salt binding, the identity of these molecular weights provide an additional assessment indicating the absence of measurable preferential hydration of LDL in KBr solutions.

[12] Molecular weight $= [6\eta s/(1 - \bar{V})^{3/2}[3\bar{V}/4]^{1/2}N$ (Schumaker and Adams, 1969, and Fisher, 1970). To correct this estimated molecular weight to the true molecular weight, the value must be multiplied by $(1.11)^{3/2}$.

Addendum

Derivation of the Equation for Calculating the Hydrated Density of LDL from Sedimentation Velocity Measurements in H_2O and in D_2O Solutions. Assume the molar volume of LDL does not change on deuteration. Let X = specific weight increment due to deuterium exchange (gram per gram of LDL). As a result the density of the deuterated protein (ρ_D) = $\rho_H(1 + X)$, where ρ_H is the density of protonated LDL, and $M_D = M_H(\rho_D/\rho_H)$.[13] Substitution into the Svedberg equation yields $(s_H = M_H/Nf)[1 - (\rho_{H2O}/\rho_H)]$, and $M_H = Nfs_H/1 - (\rho_{H_2O}/\rho_H)]$, but similarly

$$s_D = \frac{M_H\left(\frac{\rho_D}{\rho_H}\right)}{Nf}\left(1 - \frac{\rho_{D_2O}}{\rho_D}\right)$$

and substituting for M_H

$$s_D = \frac{(s_H)(Nf)\left(1 - \frac{\rho_{D_2O}}{\rho_D}\right)\left(\frac{\rho_D}{\rho_H}\right)}{Nf\left(1 - \frac{\rho_{H_2O}}{\rho_H}\right)}$$

Upon rearranging terms and substituting $\rho_D = \rho_H(1 + X)$

$$\frac{s_D}{s_H} = \frac{\left(1 - \frac{\rho_{D_2O}}{\rho_H(1 + X)}\right)\left(\frac{\rho_H(1 + X)}{\rho_H}\right)}{\left(1 - \frac{\rho_{H_2O}}{\rho_H}\right)}$$

Then multiplying the right-hand term by ρ_H/ρ_H, $(s_D/s_H)(\rho_H - \rho_{H_2O} = \rho_H(1 + X) - \rho_{D_2O}$, and rearranging terms

$$\rho_H\left[\frac{s_D}{s_H} - 1 - X\right] = \rho_{H_2O}\left(\frac{s_D}{s_H}\right) - \rho_{D_2O}$$

or

$$\rho_H = \frac{\rho_{H_2O}\left(\frac{s_D}{s_H}\right) - \rho_{D_2O}}{\frac{s_D}{s_H} - 1 - X}$$

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[13] s_H and s_D = sedimentation coefficient of protonated and deuterated LDL, respectively; M_H and M_D = molecular weights of protonated and deuterated LDL, respectively; ρ_H and ρ_D = densities of protonated and deuterated LDL, respectively; ρ_{H_2O} and ρ_{D_2O} = densities of aqueous and deuterium buffers; N = Avagadro's number; f = frictional coefficient.

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Temperature-Dependent Denaturation States of Horse and Human Ferro- and Ferrihemoglobins*

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ABSTRACT: The conformational differences that have previously been shown to exist between horse ferrihemoglobin (Hb^+) denatured by acid at 0 and 25° have been confirmed by optical difference spectra. Similar optical differences are exhibited by human Hb^+ and, as with horse Hb^+, occur only at those wavelengths (*ca.* 370 nm) at which dimerized heme manifests its presence. Carbonylhemoglobin also exhibits a temperature-dependent difference spectrum when the denaturation pH is 1.95, but not at pH 3.2. In combination with previously published data, these results imply that separation of the prosthetic group is a prerequisite for optical observation of the difference in the denatured apoprotein at the two temperatures.

It has been known for some time that ferrihemoglobin denatured by acid at 0° differs from the denatured protein formed at 25° (Beychok and Steinhardt, 1960). This conclusion was based on a detailed analysis of the large differences in intrinsic viscosities of the proteins denatured at the two different temperatures, and of the effects of ionic strength and temperature on the titration curves of the two products.

It has also been shown that the apparent energy of activation determining the denaturation rates is much smaller below 12° than above this temperature with both horse (Beychok and Steinhardt, 1960) and human (Steinhardt and Hiremath, 1967) ferrihemoglobin; a difference in the reaction paths and, therefore, in the products formed at 0° and 25° is thus suggested. Allis and Steinhardt (1969) showed that the temperature coefficient of the simultaneous unfolding of the protein and separation of the heme changes rather than that of the subsequent dimerization of the heme.

In the present investigation, we have sought to further characterize these differences by a close study of differences in the absorption spectra of the denatured products. The purpose was to contribute to an understanding of the molecular basis of the difference between the products since this difference might illuminate the nature of the interactions involved in forming the tertiary structure of the native protein.

Experimental Procedure

Materials. Horse COHb was crystallized three times from the blood of a single animal using the method of Ferry and Green (1929) as described earlier (Steinhardt *et al.*, 1966).

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