PHYSICAL CHARACTERISTICS OF HUMAN TRANSFERRIN FROM SMALL ANGLE NEUTRON SCATTERING

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ABSTRACT The technique of small angle neutron scattering has been used to determine the molecular shape, the volume, and the molecular weight of pooled human transferrin in an aqueous solution isotonic with blood. Analysis of the measurements assuming a spheroidal molecular shape indicates that an oblate spheroid with semi-axes of length 46.6 ± 1.4, 46.6 ± 1.4, and 15.8 ± 3.8Å, and a molecular volume of (144 ± 45) × 10^3 Å^3 is the best simple approximation to the shape of the transferrin molecule. The radius of gyration, R_g, determined from a Guinier plot is 30.25 ± 0.49 Å, in agreement with R_g calculated for the oblate spheroidal shape. The molecular weight is determined to be (75 ± 5) × 10^3. The shape-independent molecular volume is found to be (98 ± 10) × 10^3 Å^3. The difference in the two volumes suggests that transferrin is not a uniform spheroid but may have a more complex shape.

INTRODUCTION

Transferrin is a glycoprotein whose general function is to transport iron in the blood. The exact nature of the iron exchange mechanism between transferrin and other molecules such as hemoglobin is not known in detail, but a knowledge of molecular characteristics of transferrin, such as shape and volume, would help in understanding how these exchange processes occur. The detailed molecular structure can only be determined by diffraction measurements on the molecule in crystalized form. However, if deductions made on the basis of such a structure determination are to have biological relevance, it is crucial to know if the molecular characteristics derived in this manner are compatible with those of the molecule in its natural state (i.e., in blood). For example, the detailed structure of crystallized human hemoglobin has been determined by x-ray diffraction measurements (1), while the molecular characteristics of hemoglobin in aqueous solution were determined by small angle scattering (see reference 2 and references therein). The compatibility of these two determinations validates the deductions about the biological functions of this blood protein which were drawn from a knowledge of its detailed structure (see for example Perutz [3]).

The detailed structure of crystalized transferrin has not yet been determined, but such measurements are in progress (4). In the present paper we report small angle neutron scattering measurements (SANS) on human transferrin in an aqueous solution simulating that of blood. The results are analyzed to provide information on the size and shape of the transferrin molecule in this "natural" environment. There has been disagreement about the correct molecular weight of transferrin (5, 6, 7), and the present measurements provide a further determination of this quantity. For purposes of calibration, some measurements have

also been made on hemoglobin, and the molecular shape of this molecule determined from our analysis is compared with the results of Schelten et al. (2).

MATERIALS AND METHODS

Specimens

The specimens were made from crystalized, pooled human transferrin purchased from Sigma Chemical Co., St. Louis, Mo., and from Miles Laboratories Inc., Elkhart, Ind. Despite the fact that 17 transferrin variants are known (8), the heterogeneity expected in pooled specimens is small, since the C variant is by far the dominant one. The specimen from Sigma was a stock item while that from Miles was a special preparation and was utilized within 2 wk of crystalization. Both specimens were essentially iron-free. The Sigma samples were examined by dissolving aliquots in Tris-glycine buffer, pH 8.3, and performing gel electrophoresis on 7.5% acrylamide stained with amido-black. Only one major dark-staining band was observed. Two minor bands with weak mobilities were also present. A similar analysis was not performed on the Miles samples but within experimental error they gave the same small angle scattering results as the Sigma samples. There is a slow exchange of deuterons with hydrogen when hemoglobin is dissolved in D_2O (2). To ensure that such exchange was completed before measurements were made on the transferrin solutions, all solutions were prepared at least 24 h before their use.

The solvent used in all measurements was Ringer's salt solution. It consisted of 9 g NaCl, 0.42 g KCl and 0.25 g $CaCl_2$ with sufficient water added to make up 1 liter of solution. These salt concentrations correspond to 0.154, 0.006, and 0.002 M, respectively, where M is the molecular weight per liter. The solution had a pH of 6.7 and upon addition of transferrin increased to values typically around 7.3. Ringer's solution was used to ensure that the osmotic environment of the transferrin molecules was similar to that of blood.

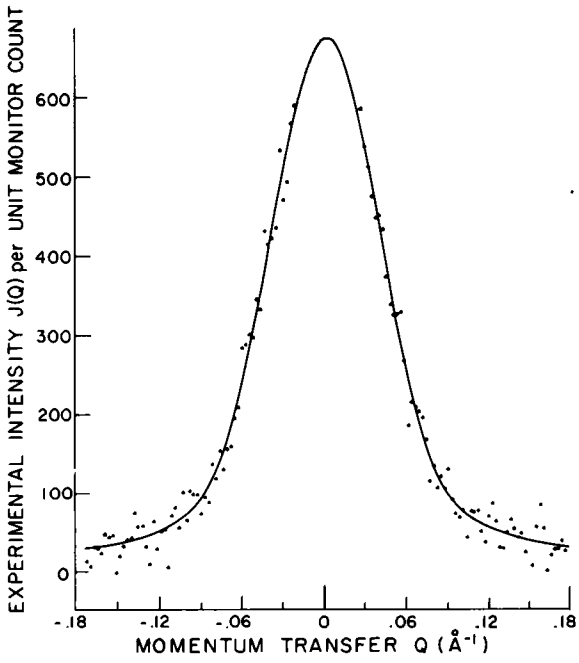

FIGURE 1 Small angle neutron scattering from transferrin (c = 4.11 g/liter) in pure D_2O Ringer's solution. The points represent the experimental data and the line is the best fit assuming an oblate spheroidal molecular shape.

Experimental Details

Measurements were made at 20°C on the C5 triple-axis spectrometer, operated in a two-axis mode, at the NRU reactor, Chalk River. The reactor spectrum was filtered by 25 cm of polycrystalline beryllium and a graphite monochromator used to obtain a monochromatic beam with wavelength, λ, of 4.085 Å and a wavelength spread ($\Delta\lambda/\lambda$) of 0.007. The monochromatic beam was analyzed by a Fermi chopper and was found to contain <0.1% of higher order contamination. The incident and scattered beams were collimated by Soller slits with angular divergences of 0.29° and 0.24°, respectively. The neutron beam was 1.25 cm wide and 5 cm high. The sample chamber was quartz glass, wall thickness 1 mm, and the specimen was 1.5 mm thick. Measurements were made by scanning the scattering angle, 2θ, in steps of 0.1°. A typical scan contained 131 steps and required 30 h running time. Repeat measurements indicated no specimen deterioration over this period.

Measurements were initially made on a sample containing 4.11 g/liter transferrin in pure D_2O Ringer's solution. This solute concentration is in the low concentration limit. It is less than twice the transferrin concentration in normal blood serum (8) and so little aggregation is expected. The results of a typical scan are shown in Fig. 1. The coherent scattered neutron intensity relative to the incoherent background scattering is very low for this transferrin concentration in solutions containing a significant proportion of H_2O. Consequently most of the experimental measurements were made with transferrin concentrations of ~40 g/liter. The results of a measurement with 38.42 g/liter in pure D_2O Ringer's solution are shown in Fig. 2. Measurements were also made with a concentration (22.2 g/liter) intermediate between the high and low values. The dependence of the SANS on the H_2O/D_2O ratio in

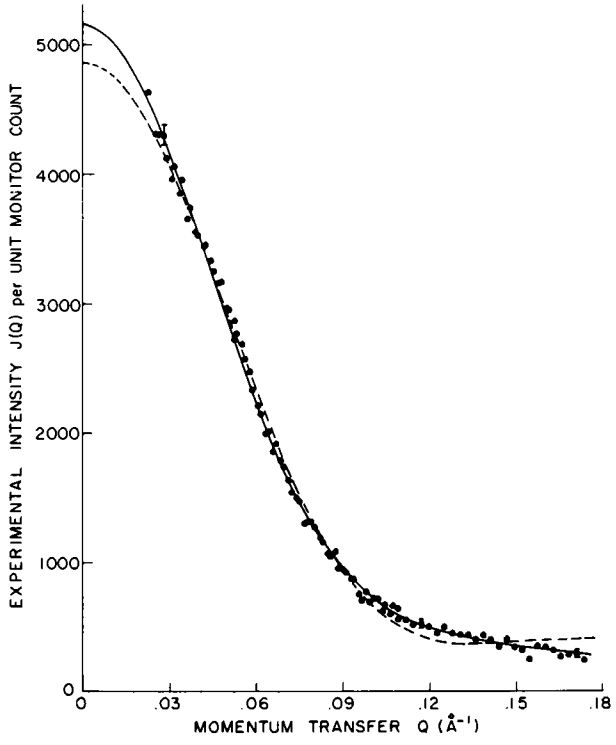

FIGURE 2 Small angle neutron scattering from transferrin (c = 38.42 g/liter) in pure D_2O Ringer's solution. The negative experimental momentum transfers are folded on to the positive axis. The solid line is the best fit to the data assuming an oblate spheroidal molecule. The dashed line is the best fit assuming a spherical molecule.

the Ringer's solution was investigated by measurements at four values of this ratio. Some measurements were also made on hemoglobin (concentration of 10.5 g/liter) in pure D_2O Ringer's solution.

ANALYSIS AND RESULTS

Molecular Shape

If $I(Q)$ is the intensity of coherently scattered neutrons at momentum transfer $Q(\equiv 4\pi \sin\theta/\lambda)$ for perfect collimation, then the experimental intensity $J(Q)$ can be written as (9):

$$J(Q) = K \int\int \{I[(Q - y)^2 + z^2]^{1/2}\} W_h(y)\, W_v(z) dy dz. \tag{1}$$

$y(z)$ is the horizontal (vertical) coordinate of a point in the detector aperture in the same units as Q; W_h, (W_v) are normalized weighting functions that specify the horizontal, (vertical) resolution functions; K is a scale factor. The function W_h was determined experimentally by scanning the detector in 2θ across the direct beam. The function could be well approximated by a Gaussian whose width (full width at half maximum [FWHM] = 0.35°) agreed well with that calculated from the known collimations. The function W_v was also assumed to be Gaussian with a width (FWHM = 3.5°) calculated from the spectrometer geometry. In practice it was found that the correction for finite experimental resolution was always small ($\simeq$1% in the molecular dimensions). The experimental intensity $J(Q)$ is given by

$$J(Q) = \left\{\frac{[S(Q) - C(Q)]}{t_S} - \frac{[E(Q) - C(Q)]}{t_E}\right\} - \delta\left\{\frac{[R(Q) - C(Q)]}{t_R} - \frac{[E(Q) - C(Q)]}{t_E}\right\} \tag{2}$$

S, R, and E are the observed intensities (normalized to constant monitor) of the sample, Ringer's solution, and empty quartz cell, respectively; t_S, t_R, and t_E are the corresponding measured transmissions; C is the observed intensity with the incident beam blocked by cadmium; δ is the volume fraction of solvent in solution.

The shape of the molecule is derived from the form of the "ideal" scattering function $I(Q)$. A functional form (not necessarily analytic) is usually assumed for $I(Q)$ and the "best" scattering function is chosen by least squares fitting to the $J(Q)$. In view of the globular shape blood proteins are thought to have, we assumed the scattering function to be that appropriate to randomly oriented ellipsoids of revolution. We write (10):

$$I(Q) = \int_0^{\pi/2} \Phi^2[QR(\sin^2\alpha + v^2\cos^2\alpha)^{1/2}] \sin\alpha \, d\alpha \tag{3}$$

$$\Phi(x) = 3\left(\frac{\sin x - x\cos x}{x^3}\right),$$

where the semi-axes of the ellipsoid are R, R, and vR. A computer program was written to convolute the scattering functions of Eq. 3 with the resolution functions W_h, W_v as described by Eq. 1. A "least squares" fit was then made to the experimental intensities of Eq. 2. The adjustable parameters were R, v and the scale factor K. A flat background was also included

as an adjustable parameter to represent any effects due to multiple scattering or scattering from small impurity fragments in the transferrin. The program evaluated the triple integral to calculate $J(Q)$ at 131 values of Q in ~5 s on a CDC Cyber 175 computer. This analysis is analogous to a procedure recently described by Sjöberg (11).

The above analysis assumes a specific shape for the transferrin molecule. However, at small Q, Guinier (10) has shown that $I(Q)$ is given by

$$I(Q) = I(0) \exp(-Q^2 R_g^2/3), \tag{4}$$

where R_g is the radius of gyration of randomly oriented molecules, independent of molecular shape. This functional form can be substituted into Eq. 1 and a resolution-corrected $I(Q)$ can then be extracted. A plot of the logarithm of $I(Q)$ vs. Q^2 is then a straight line at sufficiently small Q, and the slope of this line is related to R_g.

For the model calculation using Eq. 3 the variance-weighted least squares fit to the low transferrin concentration data in pure D_2O Ringer's solution resulted in two different minima in the "goodness of fit," χ^2. The minimum to which the fitting procedure converged was dependent on whether the anisotropy, v, was initially chosen to be greater (prolate spheroid) or less (oblate spheroid) than unity. Typical results of the fitting procedure are given in Table I. The best fit was obtained for an oblate spheroid and the line in Fig. 1 is the experimental SANS intensity calculated with values given in the upper part of Table I. However, this fit is only marginally better than that for a prolate spheroid. If the anisotropy is fixed at unity the spheroid degenerates into a sphere and the fitted $J(Q)$ with this assumption shows somewhat poorer agreement with the experimental results in the "wings" of the SANS distribution.

The R_g analysis (Eq. 4) results in the fit shown in Fig. 3. As shown by Moore et al. (12) the straight line obtained from such a plot has a slope α, which is related to the radius of gyration R_g, by the equation:

$$R_g = 0.4183\lambda \sqrt{-\alpha}. \tag{5}$$

The variance weighted least squares analysis yielded $R_g = 30.25 \pm 0.49$ Å with a χ of 0.685. From the model-dependent dimensions in the upper part of Table I, we calculate $R_g = 30.31$, 32.05, and 27.58 for an oblate spheroid, a prolate spheroid and a sphere, respectively. Thus for

TABLE I
TYPICAL RESULTS OF THE LEAST SQUARES FITTING TO SANS DATA ON TRANSFERRIN IN PURE D_2O RINGER'S SOLUTION

Concentration	Molecular shape	R (Å)	v	χ
c = 4.11 g/liter	Oblate Spheroid	46.6 ± 1.4	0.34 ± 0.07	0.668
	Prolate Spheroid	27.4 ± 1.0	2.20 ± 0.24	0.688
	Sphere	35.6 ± 0.5	1	0.751
c = 38.42 g/liter	Oblate Spheroid	42.2 ± 0.5	0.36 ± 0.03	1.02
	Prolate Spheroid	26.0 ± 0.4	1.98 ± 0.03	1.22
	Sphere	32.8 ± 0.3	1	1.81

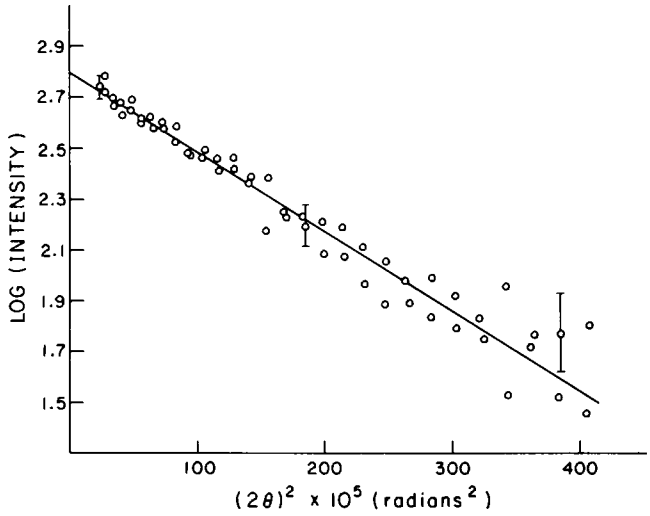

FIGURE 3 A Guinier plot of the data shown in Fig. 1. Log I is plotted as in reference 12. The straight line through the data was obtained by a variance-weighted least squares procedure.

the low concentration solution the oblate spheroid shape is favored by both the Guinier and model-dependent analyses.

The same molecular shapes were fitted to the experimental SANS from the high transferrin concentration in pure D_2O Ringer's. The results, also given in Table I, are similar to those for the low concentration data. The best fit is again obtained for the assumption of an oblate spheroid. The assumption of a prolate spheroid gives a significantly (96% confidence level) larger value of χ, while the assumption of a spherical molecule appears to be even less probable. Some results of the fitting are shown in Fig. 2. It can be clearly seen that in the "wings" of the distribution the assumption of a spherical molecule is inadequate. The fitted curve for a prolate spheroid is intermediate between those shown in Fig. 2 in the high Q range. It is known that interparticle interference effects occur at small values of Q for high concentration solutions (2). The smallest Q values in our measurements are on the border at which such effects are expected in transferrin, and the experimental intensities at the smallest Q values may be depressed because of this. Consequently, the fit to the spherical shape at small Q may be even worse than Fig. 2 indicates. The lower values of R for the high transferrin concentration (Table I) are probably due to these interference effects. By utilising R and v found for the intermediate transferrin concentration, the values for the infinite dilution limit were obtained by extrapolation. These agree with the low concentration values within experimental error. The same trends in the fitting were also observed with the R_g analysis. The data shown in Fig. 2 were analyzed with Eq. 4 and an R_g of 27.79 ± 0.17 was obtained with a χ of 1.20. From the model-dependent dimensions in the lower half of Table I, we calculate R_g = 27.54, 28.29, and 25.41 for an oblate spheroid, a prolate spheroid, and a sphere, respectively. Again the oblate spheroid is favored by both the Guinier and model calculations.

We analysed our hemoglobin measurements by means of Eqs. 1, 2, and 3 in exactly the same manner as in the analysis of the transferrin data. The differences between χ^2 for the

assumptions of each molecular shape in turn are again small. However, in contrast to the result for transferrin, a prolate spheroidal shape is marginally favored for hemoglobin. The results for the assumption of an oblate spheroid are: $R = 33.9 \pm 1.2$ Å, $\upsilon = 0.54 \pm 0.10$ with $\chi = 0.936$. Assuming a prolate spheroidal shape gives $R = 23.4 \pm 1.0$ Å, $\upsilon = 1.75 \pm 0.17$ with $\chi = 0.925$, while assuming a spherical shape ($\upsilon \equiv 1$) gives $R = 28.8 \pm 0.3$ Å with $\chi = 0.963$. Schelten et al. (2), using small angle scattering methods, determined a radius of gyration for hemoglobin of 23.7 ± 0.5. The R_g analysis of our hemoglobin results yields a value for R_g of 23.79 ± .27 Å with a χ of 0.923. The R_gs calculated from the fitted dimensions of the model shapes are 22.95, 23.55, and 22.31 for the oblate, prolate, and spherical models, respectively. Consequently the R_g analysis also indicates a prolate spheroidal shape for hemoglobin in water. The x-ray diffraction measurements of Muirhead and Perutz (1) indicated that hemoglobin was spheroidal with semi-axes of 25, 27.5, and 34.5 Å. The prolate spheroidal shape favored by our measurements is in good agreement with the molecular shape suggested by Muirhead and Perutz, although our analysis indicates the hemoglobin molecule in solution to be rather more elongated than that indicated by the x-ray diffraction results.

Molecular Weight and Volume

It can be shown (e.g., Yeager [13]) that the scattered intensity at zero momentum transfer, $I_i(0)$, is given by

$$I_i(0) = KC_i(\rho_i - \rho_s)^2 \bar{V}_i^2 M_i / N. \tag{6}$$

C_i is the weight concentration of solute molecules of species i; $\rho_i(\rho_s)$ is the neutron scattering density of the solute (solvent) molecules; $\bar{V}_i$ is the partial specific volume of the solute molecule; M_i is the molecular weight of the solute molecule; N is Avogadro's number and K a constant depending on instrumental factors. If $I_i(0)$ is measured for a "standard" molecule with the same ρ as transferrin then the molecular weight of transferrin (M_T) can be determined relative to that (M_S) of the standard.

$$\frac{M_T}{M_S} = \frac{I_T(0)\, C_S \bar{V}_S^2}{I_S(0)\, C_T \bar{V}_T^2}. \tag{7}$$

Since the blood proteins transferrin and hemoglobin are of similar volume, V, they will have similar scattering densities if the sums of their coherent neutron scattering lengths, $\Sigma_i a_i$, are similar. For a given molecule, a plot of $[I(0)/C_i]^{1/2}$ vs. the D_2O concentration in D_2O/H_2O mixtures, will give the contrast match point (2). Fig. 4 shows this plot for transferrin and gives the contrast match point as 40.5 ± 0.5% D_2O. The same value was found for hemoglobin (2). If there were no exchange this contrast match point would uniquely determine ρ_i. If exchange takes place, then ρ_i ($= \Sigma_i a_i / V$) is modified because some of the deuteriums from solution have replaced hydrogens. However, if the concentration dependence of the exchange is the same for two different molecules in dilute solution and if their ρ_i is the same at one concentration, then their p_i is the same at all concentrations.

A comparison of the amino acid compositions of transferrin (6, 8) and hemoglobin (14) shows that both molecules have similar amino acid abundances. Carbohydrates are also present in transferrin, but the percentage is small (~6%). Furthermore, for pH ~7, the

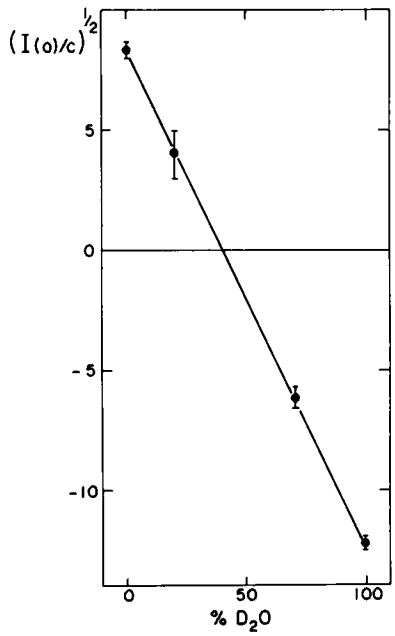

FIGURE 4 The square root of the concentration-normalized intensity at $Q = 0$ vs. the percentage of D_2O in the Ringer's solution. The intensity vanishes for 40.5 ± 0.5% D_2O.

calculated number of exchangeable hydrogens is similar for transferrin and hemoglobin. For hemoglobin it is 950 (2) and for transferrin, including carbohydrates and assuming a nominal molecular weight of ~75,000 (7), it is ~1,050. If we normalize the value of $[I(0)/c]^{1/2}$ for transferrin in pure H_2O to the corresponding value for hemoglobin (2) we find that the lines representing $[I(0)/c]^{1/2}$ vs. D_2O concentration for transferrin and hemoglobin are identical within experimental error. Consequently the concentration dependence of exchange is similar for transferrin and hemoglobin. Since the contrast match points for the two molecules are also equal, then ρ_i for the molecules are equal at all D_2O concentrations.

The partial specific volumes of transferrin and hemoglobin are known (8, 16), and we can use Eq. 7 to determine the molecular weight, M_T, of transferrin relative to that of hemoglobin. The values $I_T(0)$ and $I_S(0)$ are determined from the Guinier analysis of our data and the molecular weight of hemoglobin is taken to be 64,500 (1). After correcting this value for exchange, the molecular weight of transferrin is then found to be $(75 \pm 5) \times 10^3$, which is in fair agreement with other recent measurements (7).

It can be shown (e.g., Schelten et al. [2]) that at zero contrast the volume of the solute molecule, V, is equal to the volume of excluded solvent and is given by

$$V = \sum_i a_i/\rho_s, \tag{8}$$

where a_i is the coherent neutron scattering length of the i^{th} constituent of the solute molecule.

The composition of transferrin in terms of its amino acid and carbohydrate constituents is known in the form of weight fractions of the transferrin molecular weight (8), (6). Since the molecular weight of each amino acid and carbohydrate constitutent is known and we have

determined the molecular weight of transferrin, the number of molecules of each species can be found. The value of $\Sigma_i a_i$ can then be evaluated provided exchange is allowed for in the manner prescribed by Schelten et al. (2). In analogy with these authors we define the exchange-corrected value of this sum as $(\Sigma_i a_i)_{sol}$, where

$$\left(\sum_i a_i\right)_{sol} = \left(\sum_i a_i\right)_{H_2O} + 1{,}050\,(a_D - a_H)C_{D_2O}, \tag{9}$$

where C_{D_2O} is the D_2O fraction.

Since all our measurements commenced 24 h after mixing, we assume maximum exchange. Evaluating $(\Sigma_i a_i)_{sol}$ according to Eq. 9 we obtain a value of $2{,}188 \times 10^{-12}$ cm for transferrin. Assuming dilute solutions, the density, ρ_s, of the scattering lengths of the solvent is given by $\rho_s = \rho_{H_2O} + (\rho_{D_2O} - \rho_{H_2O})\, C_{D_2O} = 2.24 \times 10^{10}\ \text{cm}^{-2}$. If $\Sigma_i a_i$ in Eq. 8 is replaced by $(\Sigma_i a_i)_{sol}$ from above, then V is found to be $(98 \pm 10) \times 10^3$ Å^3, where the error is estimated from uncertainties in the molecular weight, the deuteration, and the composition.

DISCUSSION

The present measurements have shown the transferrin molecule to be an oblate spheroid of revolution with semi-axis of 46.6, 46.6, and 15.8 Å. The volume of this oblate spheroid is $(144 \pm 45) \times 10^3$ Å^3. From the zero-contrast point the shape-independent volume of transferrin is $(98 \pm 10) \times 10^3$ Å^3. In the case of hemoglobin our measurements suggest the molecular shape is a prolate spheroid with a volume of 94×10^3 Å. From the semi-axes quoted by Muirhead and Perutz (1) the spheroidal volume of hemoglobin is 99×10^3 Å. However, the shape independent volume determined by Schelten et al. (2) is 83×10^3 Å^3.

The shape independent volume is the smallest one which can be measured for a molecule. It is the volume which is inaccessible to the solvent. We see that for both transferrin and hemoglobin the shape independent volume is indeed smaller than that of the corresponding spheroid. For hemoglobin a large groove is known to separate the two β-chain components and this is probably the cause of the difference in the volumes. In the case of transferrin it seems reasonable to assume that a part of the difference between the two volumes is caused by a similar effect. However, in view of the rather large experimental errors we cannot be more specific about the molecular shape.

The molecular weight of transferrin is determined by the present measurement to be $75 \pm 5 \times 10^3$. Roberts et al. (5) obtained values lying between 73,200 and 76,000, which were significantly smaller than the then accepted value of 90,000. Palmour and Sutton (6) subsequently reported a value of 76,000 and more recently Lambin (7) quoted values ranging from 65,000 to 76,000. These measurements were all made by classical sedimentation or diffusion methods and the determination reported in the present paper is the first by a microscopic technique.

A dielectric molecule such as transferrin will be susceptible to electric fields generated in its environment by, for example, polar membranes. According to Landau and Lifschitz (17), if a dielectric spheroid is subjected to a uniform electric field, a torque will then be applied to the spheroid, tending to turn the symmetry axis of an oblate (prolate) spheroid perpendicular (parallel) to the field. Neglecting rotational diffusion effects, neighboring transferrin and

hemoglobin molecules in such a field would thus tend to form a particular configuration with the symmetry axes of the molecules having a definite orientational relationship.

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