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THE CONCENTRATION DEPENDENCE OF THE HEMOGLOBIN MUTUAL DIFFUSION COEFFICIENT *

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Laser correlation spectroscopy was used to measure the mutual diffusion coefficient, D, of human cyanomethemoglobin (Fe^{+++}:CN) at varying protein concentrations. These measurements were made at 20°C in a 0.1 M phosphate buffer solution at pH 7.0. For low protein concentrations we find $D = (6.43 \pm 0.26) \times 10^{-7}$ cm^2/s and that there is a near linear decrease from this value at higher concentrations. The linear relation between the diffusion coefficient and protein concentration allows us to deduce the value of the linear frictional volume fraction coefficient, $K_f = 7.75$, and to extrapolate to hemoglobin concentrations equivalent to that in the red blood cell where we estimate $D = 4.25 \times 10^{-7}$ cm^2/s. Various theoretical predictions of the dependence of the mutual diffusion coefficient on concentration are tested; we find that the generalized Stokes–Einstein relation can be made to fit our high concentration data if we assume a hard-sphere model and if we include a term involving a hydrodynamic interaction integral.

1. Introduction

We have used laser scattering spectroscopy to measure the mutual diffusion coefficient of human cyanomethemoglobin (Fe^{+++}:CN) under the condition of variable protein concentration. There are several advantages to the laser approach as opposed to the conventional measurement of the diffusion coefficient which is done by either tracer or porous membrane techniques [1]. The laser scattering method can be used over a wide range of protein concentrations, is often at least an order of magnitude faster than other methods even for weak scatterers such as hemoglobin, and can be used as an nonperturbing probe of scatterers contained in a closed system.

One of the motivating goals of this research was to determine the mutual diffusion coefficient of hemoglobin at concentrations approaching that in the human red blood cell. Such a quantity cannot be readily deduced from low concentration data and should be of considerable interest to respiratory physiologists.

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There has been some recent theoretical speculation relating the dependence of the macromolecular mutual diffusion coefficient to concentration [2–5]; however, experimental evidence dealing with this problem has been quite limited. We have attempted to provide experimental data for hemoglobin up to a macromolecular volume fraction of about 0.37. The theoretical considerations of Phillies [3–5] depend in part on empirical knowledge of the viscous frictional drag coefficient which, fortunately, can be derived from existing tracer diffusion measurements made on various forms of hemoglobin [6,7].

It is not apparent that laser light scattering spectroscopic measurements of the mutual diffusion coefficient should agree with the more conventional measurements of this same quantity. The experimental work reported in this paper shows a marked disagreement at high concentrations between the laser technique and the conventional membrane technique [6].

2. Theory

There are several review articles [8–11] dealing with the theory of laser selfbeat spectroscopy. A book pub-

lished under the joint editorship of Cummins and Pike [12] has many contributions from several authors dealing with the theoretical aspects of laser spectroscopy. We will state here only the commonly accepted results of theoretical considerations.

The observable quantity is the photocurrent autocorrelation function, $I(\tau)$,

$$I(\tau) = \langle i^*(t)\, i(t+\tau)\rangle \tag{1}$$

where $i(t)$ is the photocurrent output of a square-law detector such as a photomultiplier and where the symbol $\langle\ \rangle$ denotes a time average. The photocurrent autocorrelation function $I(\tau)$ is the Fourier dual of the photocurrent power spectral density and can be observed using a commercial correlation analyzer. An article by Kam [13] and co-workers describes the technical details of these devices.

For purposes of terminology, in referring to the homodyne case of light detection we mean that the light scattered from a system of particles falls directly onto a square-law detector with no unscattered reference beam or "local oscillator" simultaneously striking the detector. In the homodyne detection used in our experiment, the photocurrent autocorrelation function can be shown [8–12] to be related to the scattered electric field auto-correlation function $R_E(\tau)$ by

$$I(\tau) = |R_E(\tau)|^2, \tag{2}$$

where

$$R_E(\tau) = \langle E^*_{sc}(t)\, E_{sc}(t+\tau)\rangle, \tag{3}$$

and where E_{sc} is the scattered scalar electric field. If the scattered field is a gaussian random variable, it can be further shown [12] that

$$R_E(\tau) = \exp(-DK^2\tau), \tag{4}$$

where D is the mutual diffusion coefficient and where K is the magnitude of the transfer momentum vector given in turn by

$$K = (4\pi n/\lambda_0)\sin(\theta/2). \tag{5}$$

Here n is the index of refraction of the liquid in which the scatterers are suspended, λ_0 is the vacuum wavelength of light and θ is the angle between the incident and scattered light.

Observation of the appropriate parameters in eqs. (2), (4) and (5) yields the value of D, the mutual diffusion coefficient. The experimental determination of D as a function of protein concentration is the fundamental problem addressed in this paper.

Einstein [14] showed in 1905 that in the absence of macromolecular interaction, that is, in dilute solutions of particles, the diffusion coefficient of macromolecular spheres can be expressed in the form

$$D = kT/6\pi\eta a, \tag{6}$$

where k is the Boltzmann constant, T is the Kelvin temperature of the solution, η is the solvent viscosity, and a is the radius of the spherical particle. In this Einstein limit of zero protein concentration we calculate the diffusion coefficient by eq. (6) to be

$$D = 6.12 \times 10^{-7}\ \text{cm}^2/\text{s}, \tag{7}$$

where $T = 293$ K, where we have measured η in the phosphate buffer to be 1.032 cP by the Cannon–Ubbelohde method [15], and where a is taken to be 34 Å [16]. This latter value is the hydrodynamic spherical radial particle size of the dissolved hemoglobin [16]. It is difficult to determine the confidence one should place in the value given in expression (7) which is calculated from one direct and one indirect viscosity measurement under the assumption of a spherical molecular shape. An error estimate of a few percent would seem reasonable; in this spirit we estimate the error in the value of expression (7) to be about $\pm 0.20 \times 10^{-7}$ cm^2/s.

Since eq. (6) is derived for limiting cases of dilute solutions of macromolecules, hydrodynamic and direct macromolecular interactions have been neglected and, hence, no inference can be made about the dependence of the mutual diffusion coefficient on protein concentration.

Phillies [3] has pointed out that previous theories of single and many particle interaction are derived for the solvent-fixed frame whereas experiments are conducted in the volume-fixed frame. In this frame, he shows that

$$D = (\partial\pi/\partial c)_{p,T}(1-\phi)/f, \tag{8}$$

where ϕ is the macromolecular volume fraction, π is the osmotic pressure, c is the average macromolecular number density, and f is the frictional drag coefficient.

Phillies [3] refers to eq. (8) as the generalized Stokes–Einstein relation. For a macromolecular hard-sphere model and for small volume-fraction concentrations, eq. (8) can be shown to reduce to eq. (6).

The osmotic pressure π of a system of hard spheres

is given by a virial expression of the form:

$$\pi/ckT = 1 + bc + \tfrac{5}{8}(bc)^2 + 0.2869(bc)^3 + 0.1103(bc)^4 + 0.0386(bc)^5 + 0.0128(bc)^6 + 0.00404(bc)^7 + 0.00122(bc)^8 + \ldots + B_n(bc)^n + \ldots, \quad (9)$$

where

$$b = 4V. \quad (10)$$

Here V is the volume of a single hard sphere and the B_n are the virial coefficients. The first four virial coefficients of eq. (9) are given by Rice and Grey [17]. The fifth virial coefficient is given by Ree and Hoover [18]. The sixth, seventh and eighth virial coefficients of eq. (9) are calculated from an approximate expression given by Ree and Hoover [18]. Differentiation of eq. (9) gives

$$(kT)^{-1}(\partial\pi/\partial c)_{p,T} = 1 + 8\phi + 30\phi^2 + 73.45\phi^3 + 141.2\phi^4 + 237.2\phi^5 + 367.7\phi^6 + 528.9\phi^7 + 721.4\phi^8 + \ldots, \quad (11)$$

where ϕ is the volume fraction of the solute. The reason we have included so many terms in eqs. (9) and (11) is to improve the convergence of eq. (11) for large values of ϕ on the order of about 0.37. For this value of the volume fraction, the last term of eq. (11) is less than 2% of the total of other terms. We will later attempt to predict from eq. (11) the behavior of the mutual diffusion coefficient as a function of concentrations up to large volume fractions.

The theoretical dependence of the frictional drag coefficient on the macromolecular volume fraction has been considered [19] but only the first term of a power expansion has been estimated. The frictional drag coefficient is usually written as

$$f = f_0(1 + K_f\phi + \ldots), \quad (12)$$

where f_0 is the value of the frictional drag coefficient in the limit of zero protein concentration and K_f is the linear frictional volume fraction coefficient. The calculated values of K_f range from 6.55 to 7.2 [19].

Combination of eqs. (8), (11) and (12) lead to the following results:

$$D = D_0[1 + (7 - K_f)\phi + \ldots] \quad (13)$$

where we have only kept first order terms in ϕ and where D_0 is the zero concentration value of the mutual diffusion coefficient and

$$D_0 = kT/f_0. \quad (14)$$

Eq. (13) predicts a linear dependence of the mutual diffusion coefficient on the macromolecular volume fraction at least for small ϕ. As will be shown later in this paper, eq. (13) describes our experimental results well for $0.003 < \phi < 0.368$ with a value of K_f which agrees within 7% to 15% of the theoretically deduced values of this parameter.

The frictional drag coefficient f, of eq. (8) is not theoretically known beyond its first order approximation; however, as Phillies [3,4] has pointed out, this coefficient can be empirically derived from tracer experiments since the value of the tracer diffusion coefficient is inversely proportional to the drag coefficient. By referring to tracer diffusion coefficient work on various forms of hemoglobin [6,7] we can evaluate the generalized Stokes–Einstein relation, eq. (8), for the hard sphere model where eq. (11) holds and compare the predicted values with our experimental results.

The generalized Stokes–Einstein relation, is derived for the hard-sphere model to include only direct interaction effects. Phillies [5] has pointed out the possibility that hydrodynamic effects may modify the generalized Stokes–Einstein equation to a form which we write in the volume-fixed frame as

$$D = \frac{(\partial\pi/\partial c)_{p,T}(1-\phi)}{f(1-\phi G)}, \quad (15)$$

where

$$G = (3/4\pi a^3 D_0)\int d\mathbf{r}^3\, \mathbf{j}\cdot\mathbf{D}^{\mathrm{T}}(\mathbf{r})\cdot\mathbf{j} \times \{\exp[-W(r)/kT] - 1\}. \quad (16)$$

Here $\mathbf{D}^{\mathrm{T}}(\mathbf{r})$ is the cross diffusion tensor which is closely related to the Oseen tensor, $\mathbf{j}$ is a unit vector in the direction of particle current flow and $W(r)$ is the potential of average force. We rewrite eq. (16) as

$$G = (3/4\pi a^3)I, \quad (17)$$

where I is the normalized hydrodynamic integral in eq. (16). As will be seen later in this paper, the hydrodynamic correction indicated in eq. (15) will be necessary to produce agreement with our experimental results; we will then be able to evaluate G and I of eqs. (16) and (17).

Altenberger and Deutch [2] have considered light scattering from dilute macromolecular solutions taking

into consideration both direct and hydrodynamic interactions. They predict that

$$D = D_0(1 + 2\phi), \tag{18}$$

where eq. (18) is subject to the limitation that $Ka \ll 1$; in our experimental case $Ka \approx 0.06$. Altenberger and Deutch [2] make use of the cross diffusion tensor which is based on Oseen's formulation for hydrodynamic interaction as does Phillies [5]. Phillies [5] points out that the Oseen tensor is not a completely adequate characterization of hydrodynamic interactions because it does not generally include hydrodynamic backflow. Phillies' work differs from that of Altenberger and Deutch in that Phillies has modified his treatment of the Oseen tensor to take into account hydrodynamic backflow.

Anderson [20] has also predicted the concentration dependence of the macromolecular mutual diffusion; this prediction is based on a theory of hindered sedimentation which assumes that macromolecular diffusion will be the same for both the case where the driving force is the concentration gradient and where it is the gravitational gradient. Anderson suggests that

$$D = D_0(1 - \phi)^{6.5}. \tag{19}$$

This prediction is experimentally tested.

3. Method

3.1. Equipment

The scattering light source was a Spectra–Physics 50 mwatt He–Ne laser (Model 125). All components were mounted on a massive steel table which rested on shock absorbing mounts. The 6328 Å light from the laser passed through appropriate neutral density filters to be focused into a sample cell located within a thermostatically controlled bath.

Scattered light was imaged onto a pinhole in front of an RCA 7326 photomultiplier the output of which was amplified and passed into a 400-channel correlation analyzer (Saicor Model 43A) which calculated the autocorrelation function of the photocurrent.

We have included in our experimental arrangement an electronic device which we call a "stop-box". The "stop-box" is a discriminator which senses a high input signal which results from the existence of a dust particle in the scattering volume. The "stop-box" turns the correlation analyzer off until the dust particle leaves the scattering region after which correlation is resumed. In this manner, we attempted to minimize spurious dust effects. Additionally, we filtered all samples in a lamellar flow "clean" room. All filters were boiled in doubly distilled water to remove surfactants. Following a suggeston by Yeh and Britton [21], we constructed filtering apparatus that omitted the use of metal or sintered glass backing plates which can act as repositories of dust particles.

Various output devices were used in conjunction with the correlation analyzer. Ultimately, data was numerically analyzed by means of a digital computer (IBM 360/67) employing the nonlinear least squares algorithm developed by Johnson and Schuster [22].

All of our experiments were conducted at a scattering angle of 90°. This angle was chosen partly because of the simplicity of the geometry and also because of our wish to avoid accepting scattered light from possible aggregates and dust since light scattered from large particles is scattered in a more forwardly direction. According to the present state of theory [3], the mutual diffusion coefficient should not deviate from the angular dependence shown in eq. (4) for $Ka \ll 1$. Phillies [3] has pointed out that angular effects should only occur if the condition $(Ka)^2/10 \ll 1$ is violated. This criterion is concentration independent. In our case $Ka \approx 0.06$ and Phillies' criterion is well fullfilled.

3.2. Concentration measurements

We determined the concentration of our cyanomethemoglobin test samples by suitable dilution and observation of the absorption spectrum with a Cary recording spectrophotometer (Model 14). In these measurements we used a millimolar extinction coefficient of 10.9 for the ferric derivative at a wavelength of 542 nm [23]. We have taken the molecular weight of the entire hemoglobin molecule to be 64 500 [24].

3.3. Convection considerations

A serious problem in working with hemoglobin is the unwanted formation of aquamethemoglobin ($Fe^{+++}:H_2O$) which strongly absorbs light at 6328 Å. To avoid the undesired formation of this form of hemoglobin, we have chosen to use the more stable form of

cyanomethemoglobin (Fe^{+++}:CN) which is a weaker absorber of light at 6328 Å. Energy absorption can be a serious matter since it might possibly lead to convection. The presence of a convective velocity, v, will give an auto-correlation function of the form

$$R_E(\tau) = a \exp(-DK^2\tau) + b \sin K \cdot v\tau, \qquad (20)$$

where a and b are constants and where the second term shows Doppler effects of the velocity component. Although the homodyne detection scheme should not be sensitive to sinusoidal terms of the type in eq. (20), we nonetheless discarded any data which showed any recognizable sinusoidal distribution of residuals after our exponential fit. In this way we feel that we have kept convective effects to a minimum. We additionally attenuated the laser input energy to the smallest possible level consistent with signal-to-noise considerations.

3.4. Sample preparation

Because of its relative chemical stability and for the reasons stated above, we used human cyanomethemoglobin (Fe^{+++}:CN) as our scattering protein rather than any of the other heme proteins. We derived the cyanomethemoglobin (Fe^{+++}:CN) from oxyhemoglobin (Fe^{++}:O_2) which was prepared by closely following the chromatographic technique developed and reported by Williams and Tsay [25].

Cyanomethemoglobin (Fe^{+++}:CN) was prepared from the chromatographically produced oxyhemoglobin (Fe^{+++}:O_2) by three successive dialyses in the cold into 0.1 M KH_2PO_4–K_2HPO_4 buffer at pH 7.0. This phosphate buffer had 0.01 M of KCN added prior to all of the dialysis processes. Dilutions were made with the buffer from the final dialysis. The stock solution of cyanomethemoglobin (Fe^{+++}:CN) was concentrated by vacuum evaporation in the cold to about 30 g/100 ml followed by dialysis into 0.1 M phosphate buffer.

3.5. Aggregation considerations

Since the aggregation of single molecules of cyanomethemoglobin into larger groupings would alter our measurement of the mutual diffusion coefficient, serious attempts were made to minimize this possibility.

Each sample was centrifuged at 218 000 g using a Spinco–Beckman L2-658 ultracentrifuge. The samples

Table I
Translation diffusion coefficient dependence on cyanomethemoglobin concentration

Protein concentration (volume fraction)	Diffusion coefficient ($\times 10^{-7}$ cm^2/s)	Average diffusion coefficient ($\times 10^{-7}$ cm^2/s)
0.00305	6.90 6.16 6.28	6.45 ± 0.40
0.00555	6.39 6.71 6.59 6.45 6.31	6.49 ± 0.16
0.00834	6.34	–
0.00978	6.48 6.18 6.62 6.69 5.93	6.38 ± 0.32
0.0182	6.42 6.44 6.04	6.30 ± 0.23
0.0271	6.05 6.17 6.39 5.84 6.10	6.11 ± 0.20
0.0357	6.36	–
0.0535	5.94 5.67 5.60 5.57	5.70 ± 0.17
0.0562	5.93 6.08 5.65 6.05 5.67	5.88 ± 0.21
0.1156	6.69 6.44 6.16	6.43 ± 0.27
0.1860	6.45	–
0.2376	5.36 5.15 4.57	5.03 ± 0.41
0.2632	5.73 4.86 4.82 4.55	4.99 ± 0.51
0.3028	5.15 4.53 4.51	4.73 ± 0.36
0.3210	5.15 4.76 4.65 4.34	4.73 ± 0.33
0.3684	5.25 4.94	5.10 ± 0.22

were then filtered thru 0.1-μ and 0.22-μ surfactant-free Millipore filters several times. No sample was observed to have a monotonically decreasing value of the measured diffusion coefficient as time progressed as would be the case if aggregation proceded at a near steady rate.

4. Results

The results of our measurements are given in table 1. Our original unit of concentration was weight-volume percent (g/100 ml) but for purposes of comparison with theory we have changed our unit of concentration to protein volume fraction.

The first fourteen points listed in table 1 which correspond to cyanomethemoglobin volume fractions between 0.00305 and 0.00978 show no concentration effects. The value of the mutual diffusion coefficients for concentrations below a volume fraction of 0.00305 are not reported here but show the effects, we believe, of tetramer dissociation. This will be reported in another paper. The fourteen low concentration points of table 1 yield the average result given below:

$$D = (6.43 \pm 0.26) \times 10^{-7}\ \mathrm{cm}^2/\mathrm{s}. \qquad (21)$$

This is in agreement with the result calculated from eq. (6) and given in eq. (7). Our uncorrected low-concentration value for D given in eq. (21) agrees well with those values derived by other researches using laser techniques on various forms of hemoglobin [26–28]. If the results given in expression (21) is used in eq. (6) to calculate the effective spherical radius of the cyanomethemoglobin molecule, a value of 32.3 Å is obtained. This is good agreement with the value of 34 Å obtained from measurements of the intrinsic viscosity of hemoglobin and also agrees well with the values obtained by small-angle neutron and X-ray scattering [29].

A nontrivial point to be considered is the determination of the factor necessary to convert hemoglobin concentration in units of weight-volume percent (g/100 ml) into protein volume fraction. This factor F is calculated from the expression

$$F = \tfrac{4}{3}\pi a^3 N/M, \qquad (22)$$

where N is Avogadro's number and M is the hemoglobin molecular weight (64 500). Using our value of a = 32.3 Å, we calculate F = 1.322 ml/g. For dry hemoglobin this factor is 0.750 ml/g [30]. The difference between

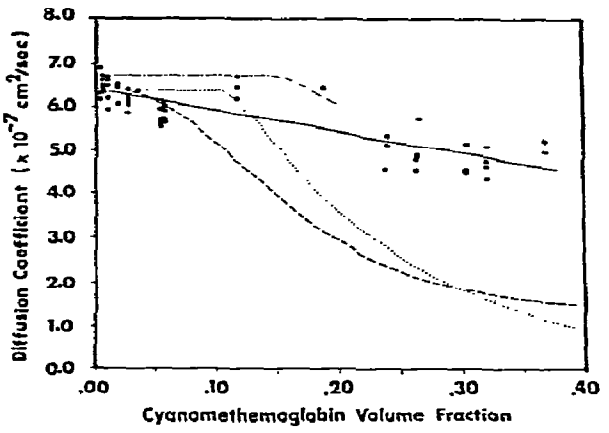

Fig. 1. The experimental values of the mutual diffusion coefficient vs. the cyanomethemoglobin (Fe^{++}:CN) volume fraction at 20°C. The indicated individual points are the results of research reported in this paper. The solid line is a linear least-squares fit of our data. The laser results of Wilson et al. [26] are shown as a dotted-dashed curve. The tracer results of Keller et al. [6] are shown as a dashed curve while those of Riveros-Moreno et al. [7] are shown as a dotted curve.

these two factors can be explained by the presence of appreciable hydration; it is easily deduced from these two values that a gram of dry hemoglobin will hydrate 0.57 g of water when in solution.

Since hemoglobin in solution carries with it significant quantities of hydrated water, the appropriate volume fraction to be used is that of the hydrated hard sphere particles. We have used the conversion factor given by expression (22) to arrive at the volume fraction concentration given in table 1. The distinction between the hydrated volume fraction and the non-hydrated volume fraction has not always been recognized.

Our results are graphically plotted in fig. 1 along with the results of Keller et al. [6], Riveros–Moreno and Wittenberg [7], and Wilson et al. [26]. Our results which are shown as individual points in fig. 1 can, by a least squares technique, be well fitted to a straight line which is shown in fig. 1 by the solid curve. The equation of the straight line of best fit is

$$D = 6.36 - 4.74\,\phi, \qquad (23)$$

where the units in eq. (23) are in 10^{-7} cm^2/s. The normalized statistical correlation coefficient for the 52 data points is 0.84 which we interpret to mean that a straight

line fit of our data is reasonably satisfactory. Eq. (23) indicates that our low protein concentration value of the mutual diffusion coefficient by a linear fit is 6.36×10^{-7} cm^2/s. This value is, of course, in agreement with the average value of the low concentration points which we have quoted in expression (21) to be $(6.43 \pm 0.26) \times 10^{-7}$ cm^2/s.

Direct comparison of eqs. (13) and (23) yields a value of $K_f = 7.75$ which compares well with the theoretical values of this quantity which range from 6.55 to 7.2 [19]. Considering that we have made use of only the first term in the power expansion of eq. (13) the agreement is surprizingly good.

We can use eq. (23) to extrapolate linearly the value of the mutual diffusion coefficient to volume fractions equivalent to that of hemoglobin in the human red blood cell. The normal hemoglobin concentration in the red blood cell is generally taken to be between 33 g/100 ml and 35 g/100 ml [31,32]. These values correspond to volume fractions between 0.44 and 0.46 and when entered into eq. (23) yield the mean mutual diffusion coefficient of 4.25×10^{-7} cm^2/s. Since the highest measurements made in our research corresponds to a volume fraction of 0.37, the linear extrapolation made here is not an extreme one.

Our results shown in fig. 1 are also plotted with those of other researchers. Wilson and co-workers [26] used a light scattering technique similar to ours to measure the mutual diffusion coefficient of oxyhemoglobin (Fe^{++}:O_2) in 0.1 M KCl at 20°C and at a pH of 7.0. Their results, shown as a smooth curve in fig. 2 for clarity, is drawn from 9 points all of which have little deviation from the plotted curve. Within the range of their measurements, the results of Wilson et al. [26] agree with our results. Riveros-Moreno and Wittenberg [7] measured the tracer diffusion coefficient using radioactive detection techniques. Their work was done on oxyhemoglobin (Fe^{++}:O_2) in 0.1 M phosphate buffer at pH 7.3. The smooth curve shown in fig. 1 was drawn by Riveros-Moreno and Wittenberg from 12 points, two of which had appreciable deviations from the smooth curve. The curve of fig. 1 attributed to Keller et al. [6] is taken only from their chemical tracer results on aquamethemoglobin (Fe^{+++}:H_2O) in a 0.1 M phosphate buffer at pH 7.3. We have modified the original concentration values given by Keller et al. [6] upwards by the ratio 12.2/10.9 which reflects the different values [33] of the ferric derivative millimolar extinction coefficient used in this work and in that of Keller et al. [6]. The individual points of the work of Keller et al. [6] deviate very little from the smooth curve of fig. 1. Keller and co-workers [6] made their chemical tracer measurements at temperatures of 25°C and 37°C. Phillies [5] has pointed out that when Keller's results are normalized to D_0, the low concentration limit of the tracer diffusion coefficient at the appropriate temperature, the results at the two temperatures fall on one curve. This is the curve we have shown in fig. 1 where we have scaled the curve to have $D_0 = 6.43 \times 10^{-7}$ cm^2/s which is our average value of the dilute mutual diffusion coefficient.

Both the tracer studies of Riveros-Moreno and Wittenberg [7] and those of Keller et al. [6] show essentially the same feature; namely that the tracer diffusion coefficient falls off more rapidly with increasing protein concentration than does the mutual diffusion coefficient as measured by laser correlation spectroscopy.

One purpose of the work by Keller, Canales, and Yum [6] was to investigate the relationship between the tracer diffusion coefficient and the mutual diffusion coefficient as measured by membrane techniques. An unexpected result of their work was that there was no appreciable difference between the two types of diffusion coefficients; these surprizing results have not yet been explained. Even more significant from the point of view of our work is that our laser measurements of the mutual diffusion coefficient do not agree with Keller's [6] membrane measurements of this same quantity. Apparently, the two different techniques do not measure the same parameter. Understanding the mechanism behind this disagreement should provide needed insight into diffusion concepts. Phillies [3] has pointed out that laser light scattering spectroscopy measures the decay of fluctuations in a single spatial Fourier component of the concentration fluctuation whereas other techniques describe a sum over the decays of the spatial Fourier components of the fluctuation. Perhaps this type of consideration may lead to an understanding of the disagreement between the laser and membrane measurements of the mutual diffusion coefficient.

We have used the reported tracer diffusion coefficient measurements on hemoglobin in deducing the concentration dependence of the generalized Stokes–Einstein relation, eq. (8). We have assumed that the tracer diffusion coefficient is inversly proportional to the frictional drag coefficient. Combining the results of tracer work with eq. (8) and (11) we are abie to calculate the con-

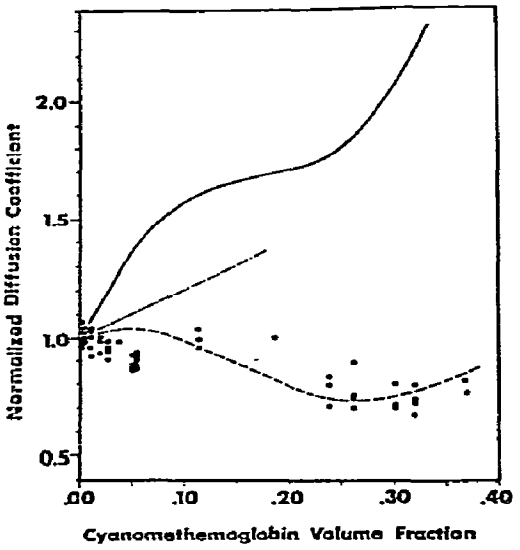

Fig. 2. Theoretical predictions of the dependence of the mutual diffusion coefficient on volume fraction. The dotted-dashed line is the prediction of Altenburger and Deutch [2]. The solid line is the generalized Stokes–Einstein relation incorporating Keller's [6] tracer data. The dashed line is the hydrodynamically modified generalized Stokes–Einstein relation. Individual points are our data.

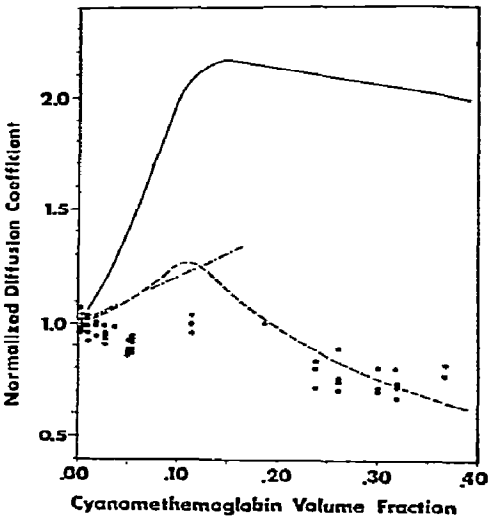

Fig. 3. Theoretical predictions of the dependence of the mutual diffusion coefficient on volume fraction. The dotted-dashed line is the prediction of Altenburger and Deutch [2]. The solid line is the generalized Stokes–Einstein relation incorporating Riveros-Moreno's [7] tracer data. The dashed line is the hydrodynamically modified generalized Stokes–Einstein relation. Individual points are our data.

centration dependence of the generalized Stokes–Einstein relation for the hard sphere model. We show the results of this type of calculation graphically in fig. 2 and fig. 3; for clarity we have chosen to display separately the work based on the chemical tracer measurements of Keller et al. [6] and that based on the radioactive work of Riveros–Moreno et al. [7]. As is readily seen from fig. 2 and fig. 3, the generalized Stokes–Einstein equation, which is derived only for direct macromolecular interaction, does not agree with our experimental results. Also displayed in figs. 2 and 3 is the theoretical prediction of Altenberger and Deutch [2] which includes hydrodynamic as well as direct interactions considerations and which is given by eq. (18); these authors limit eq. (18) to dilute solutions of macromolecules. The predictions of Altenberger and Deutch [2] do not closely agree with the experimental results.

If the generalized Stokes–Einstein relation is modified to include a hydrodynamic term as suggested in eq. (15), a measure of agreement can be achieved between theory and experiment. In the case of the theoretical predictions based on the tracer work of Keller et al. [6] the general features of the experimental data agree with the hydrodynamically corrected curve for volume fractions greater than 0.10 as is shown in fig. 2. The dimensionless hydrodynamic factor of eq. (15) which we have used for the fit shown in fig. 2 is $G = -5.80$.

A hydrodynamically modified Stokes–Einstein relation has been formulated with less success using the radioactive tracer data of Riveros–Moreno and Wittenberg [7]; this is shown in fig. 3. The fit with the experimental data is reasonable for protein volume fractions above about 0.19 but is poor below this value. The dimensionless hydrodynamic factor used in the fit of fig. 3 is $G = -5.76$ which is in close agreement with the value for the fit of fig. 2.

Using a dimensionless hydrodynamic correction factor of $G = -5.8$, we conclude the value of the hydrodynamic interaction integral in eqs. (16) and (17) to be

$$I = -8.2 \times 10^{-19}\ \text{cm}^3. \qquad (24)$$

The cube root of the magnitude of this number is 94 Å and presumably is closely related to the range of hydrodynamic forces.

Although not graphically shown, the prediction of

Anderson [20] given in eq. (19) and based on sedimentation theory is found not to agree closely with our data.

5. Conclusions

Our experimental measurements of the mutual diffusion coefficient by laser techniques show a concentration dependence that is well fitted by a linear relation, eq. (13), which is predicted by a hard sphere model. The generalized Stokes–Einstein relation which includes only direct interaction considerations does not agree with our results; however, if a hydrodynamic term is included, a fit can be forced at large protein concentrations.

Since our data disagrees with the hydrodynamically modified Stokes–Einstein equation which makes use of the tracer data of Keller et al. [6] (fig. 2) at volume fractions below about 0.20, we can deduce that the distance between hemoglobin centers for this volume fraction is about 112 Å. In the case of the work of Riveros–Moreno and Wittenberg [7] (fig. 3) where disagreement lies at volume fractions below about 0.19, we can deduce that the distance between hemoglobin centers is about 91 Å. Additionally, from considerations of the magnitude of the hydrodynamic interaction integral of eq. (24) we suspect that the range of hydrodynamic forces is about 100 Å.

We can speculate that at dilute concentrations of hemoglobin where the average separation distance between hemoglobin molecules is greater than about 100 Å, there will be little effect of hydrodynamic forces. Because of the presence of a 0.1 M KH_2PO_4–K_2HPO_4 buffer, it is very likely that direct interactions will also be negligible at low protein concentrations since the charged buffer ions will exert electrical screening effects. It seems reasonable to expect that the observed mutual diffusion coefficient will be essentially constant up to a concentration where the separation distance of the macromolecules is sufficiently small so that the direct forces will not be effectively screened and the hydrodynamic forces will be felt.

If the hydrodynamically modified generalized Stokes–Einstein relation of eq. (15) is a valid description of the concentration dependence of the mutual diffusion coefficient and if the hemoglobin molecules behave like hard spheres, we are forced to conclude from our data that direct interaction effects and hydrodynamic effects nearly cancel each other out at least over the concentration range of our experiment. This would explain the apparent success of a linear concentration dependence of the mutual diffusion coefficient as indicated in eq. (13). Whether or not the near cancellation of hydrodynamic and direct interaction effects is a fortuitous situation or whether or not it can be generalized to other systems will require further experimental work.

We have linearly extrapolated our data to protein concentrations equivalent to that in the red blood cell and predict the mutual diffusion coefficient to be 4.25×10^{-7} cm^2/s at such a concentration. This value is about 3 to 6 times larger than the tracer diffusion coefficient at the same concentration [6,7]. The difference between the mutual diffusion coefficient and the tracer diffusion coefficient can be summarized as follows: the tracer diffusion coefficient measures the flow of a labeled species in a system containing equal and opposite gradients of labeled and unlabeled particles whereas the mutual diffusion coefficient measures the flow of a species caused by a gradient in that species. Consideration of the oxygenated hemoglobin molecule diffusing in the red blood cell will be dependent on the appropriate use of the proper diffusion coefficient. Estimates of the values of both the mutual diffusion coefficient and the tracer diffusion coefficient now exist and should prove valuable in the consideration of oxygen transport across the red cell membrane.

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