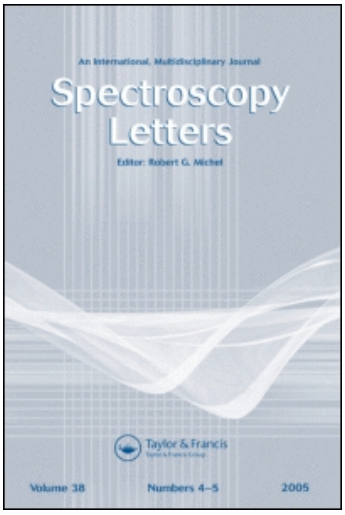


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# A Photon Correlation Study of the Concentration Dependence of Macromolecular Diffusion

Robert L. Marlowe[a]; H. E. Jackson[b]
[a] Department of Physics and Astronomy, University of Tennessee at Chattanooga, Chattanooga, TN [b] Department of Physics, University of Cincinnati, Cincinnati, OH

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# A PHOTON CORRELATION STUDY OF THE CONCENTRATION DEPENDENCE OF MACROMOLECULAR DIFFUSION

Key Words: photon correlation spectroscopy, macromolecular diffusion, polystyrene spheres



Robert L. Marlowe
Department of Physics and Astronomy, University of Tennessee at Chattanooga, Chattanooga, TN 37403

and

H.E. Jackson
Department of Physics, University of Cincinnati, Cincinnati, OH 45221-0011

## ABSTRACT

The generalized Stokes-Einstein equation, which predicts a linear dependence of the diffusion coefficient upon concentration for dilute solutions, has been investigated for highly concentrated solutions by photon correlation spectroscopy. The globular protein ovalbumin was found to exhibit a well-localized peak in its mutual diffusion coefficient at a concentration of ≈ 100 mg/ml, superimposed on a general linear trend to smaller values with increasing concentration. The appearance of the peak can be explained in a semi-quantitative manner by the Debye-Huckel theory for macro-ionic interactions.

## INTRODUCTION

In the past 25 years, the study of macromolecular Brownian motion by dynamic laser light scattering has yielded important data concerning the size, shape, structure, and modes of interaction of macromolecular systems.[1-3] The technique of photon correlation spectroscopy (PCS) has proven to be the method of choice for investigating the diffusion coefficients of large molecules. While the great majority of studies use dilute solutions (1- 5% solute concentration by weight), we wished to extend the range of the diffusion coefficient to higher concentrations (~ 25%). The purpose for doing so was to investigate the applicability of the generalized Stokes-Einstein equation in this region.



This equation predicts a linear dependence of the diffusion coefficient upon concentration (to first-order) *for dilute solutions*. However, as pointed out by Keller et al.[4], important biological processes associated with protein diffusion occur at finite, often high, regions of concentration. Examples could include the oxygen carrier transport of hemoglobin and myoglobin, the ultrafiltration of blood in the kidneys, atherosclerotic plaque formation, thrombosis, and antigen-antibody reactions. In our work, the diffusion coefficient of a typical globular protein, ovalbumin, was studied using PCS into a region of high protein concentration. Anticipating our results, we have found that the predicted linear dependence seems to persist to higher concentrations, *except* for a region where the initial assumption of particle independence, expected in dilute solutions, begins to break down.

In the first sections, we outline the theoretical underpinnings for photon correlation measurements and for the generalized Stokes-Einstein equation, respectively. We next describe the experimental technique, and the results and discussion follow.

## LIGHT SCATTERING FROM MACROMOLECULES AND PHOTON CORRELATION SPECTROSCOPY

The relation between the diffusion coefficient D and the autocorrelation function of the quasielastically scattered laser light has been derived by many researchers. We have synthesized the approaches of Berne and Pecora,[5] and Cummins,[6] in the brief overview here, so that our assumptions and approximations will be clear.



The initial experimental assumptions made for our aqueous solutions are:

i) the scattered electric field $E_S(R_o, t)$ is taken in the dipole approximation ($R_o >> r$) to be the superposition of the fields scattered from N individual solute particles in the scattering volume:

$$E_S(R_o,t) = \sum_{i=1}^{N} A_i(R_o,t) e^{i(\mathbf{q}\cdot\mathbf{r}_i(t)-\omega_o t)}, \qquad (1)$$

where $A_i(R_o, t)$ is the amplitude of the field scattered from the ith particle, whose center of mass is located at $\mathbf{r}_i$; $R_o$ is the distance from the sample to the detector; $\mathbf{q} = \mathbf{k_s} - \mathbf{k_o}$, i.e., $\mathbf{q}$ is the usual scattering vector, and $\omega_o$ is the angular frequency of the incident laser light.

ii) in specifying $E_S$ to have the simple form of Eq. (1), we are excluding multiple scattering effects, and assuming that each

scatterer interacts independently with the incoming electric field. Such conditions are easy to arrange with sufficiently dilute solutions. iii) we shall not consider the depolarized component of the scattering. iv) we shall treat the scatterers as identical spherical particles *whose positions are uncorrelated*, and whose average polarizability is time-independent.

For such a system of N particles in the scattering volume, we invoke the reasonable assumption of stationarity for $E_s$, and write the electric field autocorrelation function as

$$G^{(1)}(t) \equiv \left\langle E_s^*(0)E_s(t)\right\rangle$$

$$= \left\langle \sum_{j=1}^{N} A_j^*(R_o,t)e^{\left(-i\mathbf{q}\cdot\mathbf{r}_j(0)\right)} \sum_{i=1}^{N} A_i(R_o,t)e^{\left(i\mathbf{q}\cdot\mathbf{r}_i(t)\right)} \right\rangle e^{-i\omega_o t} \qquad (2)$$

Using assumption (iv) above, we can separate the orientational (amplitude-dependent) and translational parts of the field dependence. Cross-terms in the translational part can be shown to be proportional to $\delta(\mathbf{q})$. Since our light scattering experiments are not performed in the forward ($\mathbf{q} = 0$) direction, we are assured that such cross-terms will not contribute to the ensemble average in Eq. (2), which then simplifies to

$$G^{(1)}(t) = N|A|^2 \left\langle e^{-i\mathbf{q}\cdot[\mathbf{r}(0)-\mathbf{r}(t)]}\right\rangle e^{-i\omega_o t} \qquad (3)$$

To evaluate the ensemble average, we use the conditional probability $G_S(\mathbf{R},t)$. Letting $\mathbf{r}(0) - \mathbf{r}(t) = \mathbf{R}$, we define $G_S(\mathbf{R}, t)d^3R$ to be the probability that a particle initially located at the origin will be found at time t in a region $d^3R$ centered about $\mathbf{R}$. Then,

$$\left\langle e^{-i\mathbf{q}\cdot\mathbf{R}}\right\rangle = \int d^3R\, G_s(\mathbf{R},t)e^{-i\mathbf{q}\cdot\mathbf{R}} \equiv F_s(\mathbf{q},t). \tag{4}$$

In light of its definition, it is reasonable to believe that $G_s(\mathbf{R},t)$ constitutes a solution to the diffusion equation, i.e.,

$$\frac{\partial G_s(\mathbf{R},t)}{\partial t} = D\nabla^2 G_s(\mathbf{R},t). \tag{5}$$

A spatial Fourier transform of Eq. (5) leads to the solution

$$F_s(\mathbf{q},t) = e^{-Dq^2t}. \tag{6}$$

This provides the desired overview, with our assumptions identified, connecting the autocorrelation function of the scattered field with the diffusion coefficient of the Brownian particles:

$$G^{(1)}(t) = N|A|^2 e^{-Dq^2t} e^{-i\omega_o t}. \tag{7}$$

The most efficient use of light scattering data in experimentally obtaining such an autocorrelation function is done through photon counting, in which the digital electronic pulses due to individual photons arriving at a photomultiplier are directed into an autocorrelator. Assuming that the scattered field is stationary and ergodic, the normalized "photocount" autocorrelation function is simply equivalent to the normalized intensity autocorrelation function:[8-11]

$$g^{(2)}_{pc}(m\Delta T) = \frac{< n(0)\, n(m\Delta T) >}{< n >^2} = \frac{< I(o)\, I(m\Delta T) >}{< I >^2}$$

$$= 1 + e^{-2Dq^2t}, \tag{8}$$

where n(t) is the number of photons arriving at the photodetector during an interval $\Delta T$ centered at time t, and m is a non-negative integer which specifies how many intervals have passed since t = 0. The last equality in Eq. (8) is due to the Siegert relation,[10,12,13] which connects the (Gaussian) field and intensity autocorrelations. By measuring $g^{(2)}_{PC}(t)$, one can extract D through curve fitting, since **q** is a known quantity. With the exception of one experimental detail (use of the "clipped" mode), this provides our theoretical background connecting macromolecular diffusion with the photocount autocorrelation function.



## THE GENERALIZED STOKES-EINSTEIN EQUATION

Derivations of the generalized Stokes-Einstein equation (GSE), which relates the mutual diffusion coefficient D to solute concentration $c_S$, can be found in several sources.[14-16] We use the arguments of Berne and Pecora[14] to justify the linear dependence of D upon $c_S$. When conditions are restricted to a simple 2-component, isothermal, isobaric system, the GSE can be written as

$$D = \frac{c_S}{N_A f_S}\left[\frac{\partial \mu_S}{\partial c_S}\right]_{P,T}(1-\varphi_S), \qquad (9)$$

where $c_S$ = solute concentration (moles/cm$^3$); $\mu_S$ = solute chemical potential; $\varphi_S = v_S c_S$, the solute volume fraction ($v_S$ is the partial molar volume); $f_S$ = the concentration-dependent frictional drag coefficient of the solute molecule (the frictional force on the molecule is $\mathbf{F_f} = -f_S\mathbf{v}$); and $N_A$ = Avogadro's number.

For dilute solutions, we have (to first order in $c_s$),[14]

$$\left[\frac{\partial \mu_s}{\partial c_s}\right]_{P,T} \approx RT(1 + K\varphi_s), \tag{10}$$

where K is a dimensionless constant related to the shape of the molecule. The friction coefficient $f_s$ can also be expanded in terms of $c_s$; to first order, we have

$$\frac{1 - \varphi_s}{f_s} \approx \frac{1}{f_{so}}(1 - K_f \varphi_s), \tag{11}$$

where $f_{so}$ is the friction coefficient in the limit of an infinitely dilute solution, and $K_f$ is a constant. Substitution of Eqs.(10) and (11) into (9) produces

$$D = \frac{k_B T}{f_{so}}(1 + (K - K_f)\varphi_s) + O(\varphi_s^2). \tag{12}$$

This gives the prediction of linear dependence of D upon $c_s$ (expressed in terms of the parameter $\varphi_s$). The slope (positive or negative) of this variation will depend upon solution properties such as pH and ionic strength.

The primary assumption involved in arriving at Eq. (12) is that the solute particles do not interact with each other in a system undergoing binary diffusion. The fact that our real experimental solvent consists of not only water, but also various salts and salt ions, is not expected to disturb the system. Indeed, the addition of low molecular weight salts is expected to provide counter-ion

shielding for the protein molecules against Coulomb interactions,[17] and so keep them "independent." This assumption will be examined again in the discussion.

## EXPERIMENTAL METHOD

The complete experimental details of our dynamic light scattering investigation are provided elsewhere;[18] the salient points are given below.

The $TEM_{00}$ beam ( $\lambda = 514.5$ nm) from an argon-ion laser was focussed onto the sample solution, and the scattered light detected by a photomultiplier tube. Care was taken (1) to prevent any stray light from the incident beam from reaching the detector, and (2) to maintain a good signal-to-noise ratio by keeping the coherence area[19] of the scattered light significantly larger than the illuminated area of the detector's photosensitive surface. The first of these precautions insures that our experiment measures only the homodyne ("self-beating") auto-correlation, instead of the heterodyne autocorrelation. The photon counting electronics are standard, with the exception of the photon correlator, which is of our own design.[18] All experiments were conducted at room temperature.

The autocorrelator was used in the "single-clipped" mode,[12] producing a correlation function defined as

$$G_k^{(2)}(t) = \langle n_k(0)n(t)\rangle, \tag{13}$$

where $n_k(t) = 1$ for $n(t) > k$, and $n_k(t) = 0$ for $n(t) \leq k$. The integer k is called the "clipping level," and is chosen to be close to $\langle n\rangle$, the

average number of counts per sample time interval. The single-clipped photocount autocorrelation is related to the true field autocorrelation by

$$g_k^{(2)}(t) = \frac{G_k^{(2)}(t)}{\langle n\rangle\langle n_k\rangle} = 1 + \beta\left|\frac{G^{(1)}(t)}{\langle I\rangle}\right|^2 . \qquad (14)$$

ß is a constant which contains no time dependence, and is treated as an adjustable parameter in the curve-fitting process.

In order to insure that the apparatus was working as expected, initial experiments were conducted with dilute aqueous solutions of polystyrene spheres[20] of radius 0.085 ± .005 μm (determined by electron microscopy). We made several runs at scattering angles of 110°, 90°, 75°, 60°, and 30°. An average of the radii (determined from $D = k_B T/6\pi\eta r$) taken from 22 experimental runs (four each for angles 60°-110°, six runs for 30°) yielded a radius 0.0837 ± .0023 μm. These fits were done using a single exponential fit to the data. Figure 1 shows autocorrelation data typical for all such runs with these spheres.

A semi-log plot of $(g_k^{(2)}(t) - 1)/\beta$ vs. $2q^2t$ should cause the data for different scattering angles to fall on the same line. Figure 2 shows that the data indeed scales as expected. The homodyne exponential integral fit allows for the presence of a small, but non-negligible, amount of polydispersity due to contaminants in the solution.[21] (Both the standard homodyne and the homodyne exponential integral fits used two adjustable parameters). For the same 22 runs discussed above, the average sphere radius using the

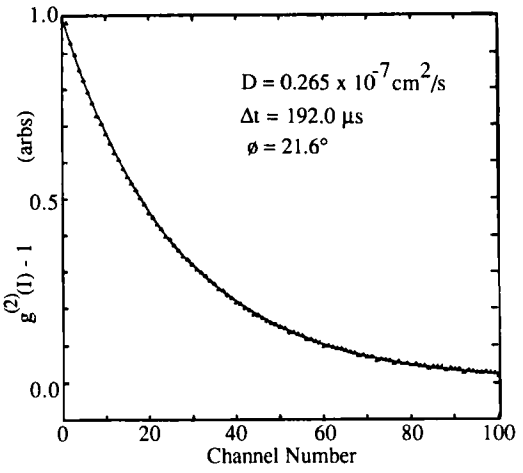



FIG.1 Normalized autocorrelation data as a function of channel number for dilute solutions of polystyrene spheres. The external scattering angle is 30°; the true scattering angle (in the medium) is shown on the diagram. The Ith channel number is converted into a time through the product IΔt.



homodyne exponential integral fit was 0.0853 ± .0028 μm. Thus, there was little if any difference between the two fitting procedures for the polystyrene sphere solutions. Our results confirmed that the experimental apparatus was performing satisfactorily.

Ovalbumin solutions were prepared using an acetate buffer,[22] then centrifuged for 3-4 hrs. at 149,000 g; the clear supernatant was then diluted to the desired concentration. Our sample solutions were adjusted to a pH of 6.30 ± .05. The solutions were filtered to remove any contaminants and/or aggregates which were larger than 0.2 μm,

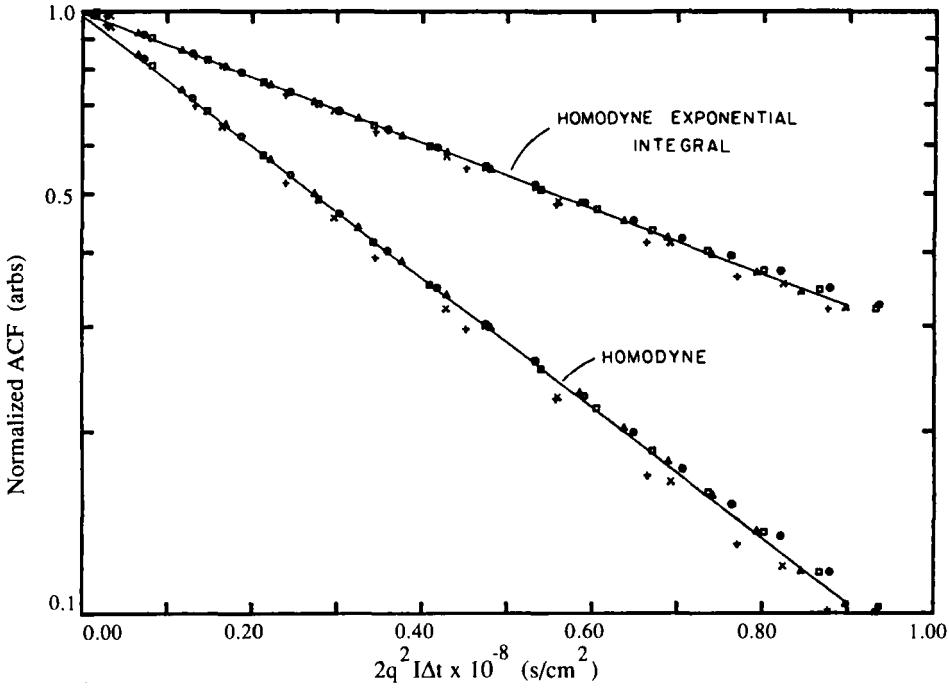



FIG.2 Semilog plots of the normalized autocorrelation function for polystyrene spheres. For the homodyne exponential integral plot, the absscissa is $q^2I\Delta t \times 10^{-8}$ s/cm$^2$, and all abscissa values are to be divided by 2. Each point represents an average, as follows: ( ) 4 runs at 110°; (Δ) 2 runs at 90°; (+) 3 runs at75°; (x) 3 runs at 60°; (o) 5 runs at 30°. The channels are given in increments of 4, beginning with I = 1. The ordinate for each plot is such that the slope of the line is -D.

and the concentrations were determined from index of refraction measurements made with an Abbe refractometer, having a precision of ±.0002.

Since PCS is relatively insensitive to macromolecular polydispersity,[23] independent checks of the solutions were necessary to

ascertain the monomericity. A dilute solution (5 mg/ml) was checked by means of Schlieren photography, and a sedimentation coefficient of 3.64 $x10^{-13}$ s calculated from these measurements. This value compares well with that of 3.66 $x10^{-13}$ s, determined by earlier researchers to represent ovalbumin at infinite dilution.[24] A concentrated solution (100 mg/ml) was checked using centrifugation through a sucrose density gradient; the final solution was scanned by an absorbance monitor at 280 nm. The resulting plot of relative protein concentration vs. position showed no visible asymmetries. Taken together, these studies provide support that we have prepared reasonably monomeric ovalbumin solutions using the procedure given above.



## RESULTS AND DISCUSSION

Photon correlation experiments on ovalbumin solutions of varying concentration were performed to determine the mutual diffusion coefficients. These coefficients were determined using the homodyne exponential integral fitting procedure to the data, which provided a somewhat better fit in the transitional region of the decaying exponentials than did the standard homodyne fit. The good quality of the fits obtained for dilute, intermediate, and very concentrated solutions is typified by the plots seen in Fig.3. All of the runs were done at an external scattering angle of 30°.

The experimental dependence of the mutual diffusion coefficient upon ovalbumin concentration is given in Fig.4. The two main features of the data seen here are clearly a general tendency of the diffusion coefficient to decrease as concentration increases, and a region

around c ≈ 100 mg/ml where D experiences a definite, but relatively narrow, rise. The first feature can be explained on the basis of the GSE (at least out to where the rise begins), since a linear dependence of D upon c is set out in Eq. (12). We note that this "dilute" behavior of D extends to roughly 10% of the solute concentration by weight for ovalbumin.

The peak in the data suggests that we explore some new ideas, e.g.:

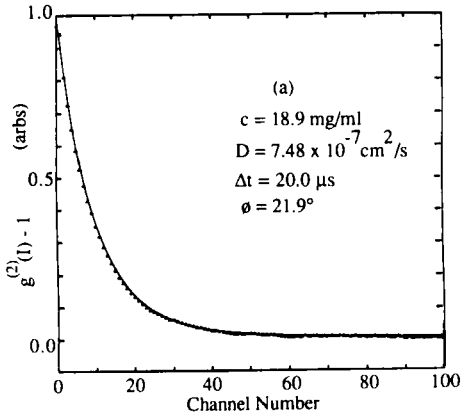
(1) the ovalbumin molecules might undergo conformational changes in this region, becoming first more and then less compact as D rises and falls;

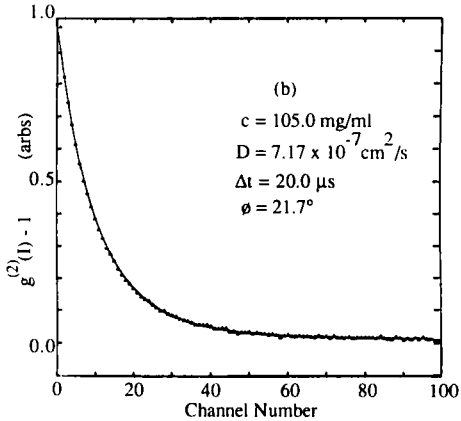
(2) the molecules might begin to communicate with each other via electrostatic interactions, and thus should no longer be regarded as independent Brownian particles; and

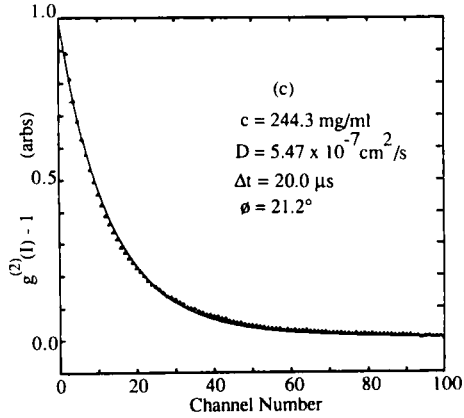
(3) the solution may be involved in some collective phenomena.

We address each of these ideas below.

In view of the facts[25] (a) that globular proteins as a group tend to have compact, symmetrical, and rigid structures which are maintained in solution, (b) that ovalbumin in particular does not appear to change its size or shape as the solution pH is varied from 3 to 12, and (c) that ovalbumin can acquire a large amount of charge before expanding, we will set our first explanation aside. Likewise, collective phenomena would result in a more ordered (perhaps lattice-like) arrangement for the solute; it is easy to argue that D would become markedly slower as a result, but not clear as to what would account for an increase in D. We therefore pursue the second suggestion stated above.



We begin by giving an estimate of the intermolecular spacing for the ovalbumin at 100 mg/ml; this is on the order of $(N/V)^{-1/3} \approx 9$ nm. The diameter of the molecule[25] is about 5.8 nm, and so we expect the surfaces of neighboring molecules to lie, approximately, about a *molecular radius* away. Next, we note that the addition of an electrolyte (e.g., NaCl) to protein solutions is commonly used to provide a means of counter-ion "screening" of the long-range electrostatic forces between the large solute macro-ions.

The theory most commonly used to describe electrostatic interactions among charged particles in solution is the Debye-Huckel theory, which develops a parameter $\kappa^{-1}$ as the screening length of the counter-ion atmosphere surrounding the solute particles. The screening parameter is defined as

$$\kappa^2 = \frac{4\pi}{\varepsilon} \frac{1}{k_B T} \sum_i z_i^2 c_i \ , \qquad (15)$$

where $\varepsilon$ ($\approx 80$) is the dielectric constant of the solvent, $z_i$ is the charge (in e.s.u.) on the ith species of ions, and $c_i$ is the number density (in $cm^{-3}$); the units of $\kappa^2$ are $cm^{-2}$. The sum goes over all ions present in the solution; we have calculated[26] that for our solutions, $\kappa^{-1} \approx 0.7$ nm.

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FIG.3 Examples of the homodyne exponential integral fits for the normalized ACF for ovalbumin, for different concentrations: (a) dilute (18.9 mg/ml), (b) concentrated (105 mg/ml), and (c) extremely concentrated (244.3 mg/ml). External scattering angle is 30°. Solid lines are the exponential least squares fits.

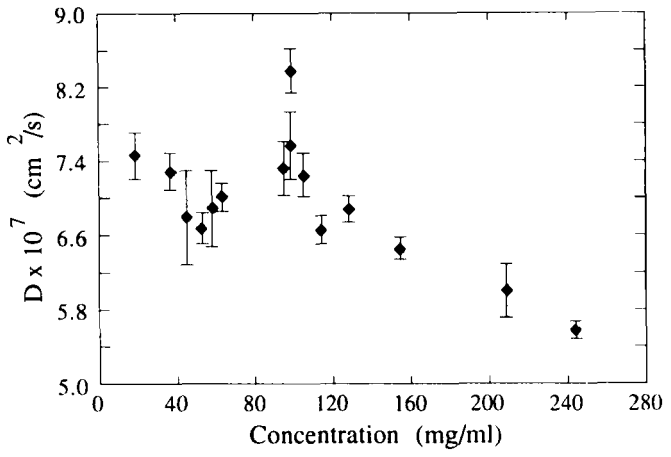


FIG.4 Mutual diffusion coefficient as a function of solute concentration for ovalbumin. Each point is an average of between 8 and 14 experimental runs; error bars represent the standard deviation of the runs.



These first-approximation results show that, on average, the ovalbumin molecules should be effectively screened from each other. However, because concentration fluctutations are present, one can ask how D is influenced by molecules which do succeed in interacting. Stephen[27] predicts that the repulsive potentials set up between the macro-ionic atmospheres will serve to *increase* the measured diffusion coefficient. However, as the ovalbumin concentration increases, it seems likely that each particle will become hindered by its neighbors in its diffusive motion, resulting in a decrease in D. How much of this decline in D is due to (a) a simple excluded volume effect, (b) electrostatic trapping, or (c) cooperative phenomena cannot be determined by our experiments.

In order to estimate the value of the enhancement in D predicted by this theory,[27] we turn to an anaylsis of the microscopic concentration fluctuations. The form of the ACF of the concentration fluctuations in q-space is expressed[28] as

$$< \delta c_1^*(q,0)\, \delta c_1(q,t) > = <|\delta c_1(q,t)|^2 > e^{-q^2 D_s t}, \qquad (16)$$

$$\text{where} \quad D_s = D_1\left[1 + \frac{q_1^2}{q^2 + \left(q_o^2 - q_1^2\right)}\right] \qquad (17)$$

Here the inverse screening parameter resulting from the ith ionic species is defined as $q_i^2 = (4\pi/\varepsilon)(1/k_B T)\, z_i^2 c_i^\circ$; $q_o^2 = \Sigma\, q_i^2$ is the sum over <u>all</u> ionic species; $c_i^\circ$ is the equilibrium number concentration, and the subscript 1 refers to the ovalbumin macro-ion. $D_1$ is the diffusion coefficient of the *non-interacting* ovalbumin molecule. We see that the observed mutual diffusion coefficient $D_S$ given by Eq. (17) is enhanced, and should (ideally) exhibit a q-dependence. The physical reason for the enhancement is that whenever the counter-ions surrounding the ovalbumin experience a repulsive force due to a neighboring counter-ion cloud, they tend to drag the macromolecule with them.[29]

There are two difficulties in providing a detailed calculation for $D_S$: the lack of good information on the net average charge of ovalbumin, and the appropriate value to use for $D_1$. For pH 6.3, at a buffer ionic strength of 0.10, the net charge of ovalbumin has been determined by electrophoresis[25] to be ≈ -7 e.s.u. We will assume this value for our solution, where the buffer strength is ≈ 0.15. $D_1$ is

concentration-dependent; if the slope of the initial concentration region (from 10 - 70 mg/ml) is extended into the region of the peak, we should expect $D_1$ to be represented by $\approx 6.50 \times 10^{-7}$ $cm^2/s$. We find then that $q_1^2 \approx 5.8 \times 10^{13}$ $cm^{-2}$. We can safely neglect the q-dependence in $D_S$, since, in general, for visible quasi-elastic light scattering $q^2 \approx 10^{10}$ $cm^{-2}$. Our expression for $D_S$ then simplifies to

$$D_S \approx (6.50 \times 10^{-7})\left[1 + \frac{5.8 \times 10^{13}}{(21.9 - 5.8) \times 10^{13}}\right] \approx 8.8 \times 10^{-7} cm^2/s, \quad (18)$$

which compares well to the value of the peak as observed.

## CONCLUSION

The present study has provided measurements of the mutual diffusion coefficient, D, of a weakly acidic aqueous solution of the globular protein ovalbumin over a wide range of concentrations (from $\approx$ 2% to 25% solute by weight). The linear variation of diffusion with concentration, predicted by the generalized Stokes-Einstein equation for non-interacting particles, is supported by our experiment up to a solute concentration of roughly 75 mg/ml. A well-defined peak in the mutual diffusion coefficient is found at a concentration of $\approx$ 100 mg/ml. The value of D in the region of the peak can be explained in a semi-quantitative manner by application of a theory for macro-ionic interactions.

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