Concentration dependence of translational diffusion coefficients for globular proteins

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This investigation examines published results of traditional diffusion experiments on ovalbumin and bovine serum albumin to determine the extent to which assumed concentration independence of the translational diffusion coefficient is a reasonable approximation in the analysis of boundary spreading in sedimentation velocity experiments on proteins. Although significant positive concentration dependence of the diffusion coefficient for both proteins is predicted by current theories, none has been detected in these experimental diffusion studies performed under the constraints of constant temperature and solvent chemical potential (those also pertinent to sedimentation velocity). Instead, the results are better described by the relatively minor concentration dependence predicted by considering solution viscosity to be an additional source of $D-c$ dependence. Inasmuch as the predicted variation in $D$ for solutions with concentrations below 10 mg mL$^{-1}$ is within the uncertainty of experimental estimates, these findings support use of the approximate solution of the Lamm equation developed by Fujita for the quantitative analysis of boundary spreading in sedimentation velocity experiments on proteins.

Introduction

The combination of a new-generation analytical ultracentrifuge and the massive advances in computer technology has rekindled interest in the quantitative description of the concentration dependence of translational diffusion coefficients ($D$) for proteins - a topic that has attracted only spasmodic attention in the past five decades. At that stage Fujita$^{1,2}$ had reported an approximate analytical solution of the continuity equation describing solute migration in a centrifugal field$^4$ that took into account the boundary sharpening that arises from negative, linear concentration dependence of the sedimentation coefficient for a system with $D$ independent of solute concentration ($c$), which is a realistic situation inasmuch as the neglect of $D-c$ dependence seems to be a reasonable experimental approximation. However, the situation is less satisfactory from a theoretical viewpoint in that the predicted positive concentration dependence of $D$ contradicts observations of slight negative $D-c$ dependence$^{7-10}$ when experiments are conducted under the constraints of constant temperature and solvent chemical potential - the conditions relevant to sedimentation velocity experiments. This investigation attempts to resolve that disparity between theory and experiment.

Theoretical considerations

Although concentration dependence of the translational diffusion coefficient for macromolecular solutes has received considerable theoretical scrutiny, much of that attention has been directed towards the description of diffusion in quasielastic lights scattering spectroscopy (dynamic light scattering) studies$^{6,11-16}$ where the solute chemical potential is being monitored under the constraint of either constant pressure$^{17-19}$ or constant volume$^{20}$ either option applies to the incompressible solutions being considered here. As noted by Phillips,$^{17}$ the disparate theoretical description of $D-c$ dependence reported by Batchelor$^4$ refers to the situation involving flux of solute molecules under the influence of an applied force, the gradient in solute chemical potential under the constraint of constant solvent chemical potential - a constraint that allows all buffer and small electrolyte components to be regarded as part of the solvent.$^{21-23}$ This simplification does not apply to light scattering experiments, where the constraint of constant pressure (or volume) dictates the consideration of these small components as additional cosolutes.$^{17-19}$

The most detailed consideration of the effects of hydrodynamic interaction on Brownian diffusion involving net flux of solute appears in the above-mentioned article by Batchelor,$^4$ which provides an alternative derivation of the standard expression for independent translational diffusion of molecules,

$$D = \frac{RT}{N_A \delta \pi \eta a}$$

(1)
where the diffusion coefficient is described in terms of the hydrodynamic (Stokes) radius $a$ and the dynamic viscosity of the medium $\eta; N_A$ is Avogadro’s number. This expression for ideal diffusion was developed initially by Einstein on the basis of the osmotic pressure of the system, and hence on thermodynamic considerations of solute chemical potential under the constraints of constant temperature and solvent chemical potential also seem to apply. These are certainly the constraints that apply in traditional diffusion experiments involving an initially sharp boundary between protein solution and the diffusate against which it has been extensively dialyzed. Furthermore, a similar situation applies to boundary spreading in sedimentation velocity studies, where the constraints of constant temperature and solvent chemical potential also seem to apply.\textsuperscript{27-29}

### An expression for $D-e$ dependence of based on thermodynamic considerations

Attempts to incorporate the effects of solute concentration into eqn (1) followed the Einstein line of reasoning by regarding the nonideality as a thermodynamic problem. On the grounds that the driving force for diffusion is the gradient in solute chemical potential the diffusion coefficient, $D$, has been expressed as

$$D = \frac{M}{N_A} \left( \frac{\partial II}{\partial e} \right)_{T,\mu_i} = \frac{RT(1 + 2BMc + ...)}{N_A}$$

where the second representation of $D$ follows from the standard virial expansion of osmotic pressure under the same constraints,

$$\left( \frac{II}{RT} \right)_{T,\mu_i} = e/M + BMc^2 + ...$$

in which $B = B_{22}/M^2$ is the osmotic second virial coefficient for solute self-interaction expressed in the usual experimental units of mL mol$^{-1}$ rather than its molar counterpart ($B_{22}$). Upon introducing the relationship

$$f = f^0(1 + k_De)$$

for the concentration dependence of the translational friction coefficient, eqn (2) becomes

$$D = \frac{RTN_Af^0(1 + k_De)}{N_Af^0}$$

By defining $D^0$ as $RT/N_Af^0$ and expressing the concentration dependence of the diffusion coefficient as

$$D = D^0(1 + k_tDc)$$

it follows from eqn (5) that

$$k_D = 2BM - k_a$$

Harding and Johnson provided two expressions for $k_D$; eqn (7) above, which corresponds to sedimentation coefficients corrected for solution density, and another expression for sedimentation coefficients corrected for solution density in which an extra term ($-\bar{v}$) appears. The appropriate correction (for solvent density) was first recognized by Fujita who amended the basic sedimentation equilibrium equation to take into account the concentration dependence of solution density—a parameter originally regarded as a constant in the integration of the buoyancy term. A consequence of this amendment was the erroneous consideration of the experimental second virial coefficient as $(2BM - \bar{v})$ until the existence of a second (compensating) error in the original basic sedimentation equilibrium equation was detected.\textsuperscript{26,32,33}

In statistical-mechanical terms the osmotic second virial coefficient for protein self-interaction may be calculated by assuming spherical geometry for the hydrated solute (radius $a$) with net charge $Z$ spread uniformly over its surface. Specifically, the expression for $B_{22}$ comprises two terms: a relatively simple term for the covolume of two identical impenetrable spheres, and a term to cover the potential-of-mean-force, $u_{ij}(x)$ between charged pairs of molecules (i and j) that are separated by distance $x$. Specifically,

$B_{22} = \frac{16\pi N_Aa^3}{3} - 2\pi N_A \int_0^\infty f_{ij}(x)x^2dx$  \hspace{1cm} (8a)

$$f_{ij}(x) = \exp[-u_{ij}(x)(kT)] - 1$$  \hspace{1cm} (8b)

in which $k$ is the Boltzmann constant; Avogadro’s number is introduced to convert $B_{22}$ from a molecular to a molar basis. The potential energy of the two molecules, $u_{ij}(x)$, can be calculated from the expression

$$u_{ij}(x) = \frac{1000Z^2/x^2(-\kappa(x - 2a))}{8\pi N_AI(1 + \kappa a)^3x} x \gg 2a$$

where $\kappa$, the Debye–Hückel inverse screening length (cm$^{-1}$), is related to the molar ionic strength of the solvent ($I$) by the expression $\kappa = 3.27 \times 10^7/\sqrt{I}$. The factor of 1000 in eqn (9) takes into account the units of ionic strength (mol L$^{-1}$) in an expression where the unit of volume is cm$^3$.

### An expression for $D-e$ dependence based on hydrodynamic considerations

From hydrodynamic considerations of the concentration dependence of the translational diffusion coefficient for a rigid spherical particle in sedimentation velocity experiments (which also involve a gradient in solute concentration), Batchelor has derived the relationship [see eqn (6.10) and (6.12) therein]

$$D = \frac{(1 + (8\phi - 6.55\phi)RT)}{N_A\bar{v}\eta_0\phi}$$

which, after replacement of the volume fraction of solute ($\phi$) by the product $v_c\phi$, becomes

$$D = \frac{(1 + (8\phi_v - 6.55\phi_v)c)RT}{N_A\bar{v}\eta_0} = \frac{(1 + (8\phi_v - k_v)c)RT}{N_A\bar{v}\eta_0}$$

where the alternative formulation takes advantage of the Batchelor expression for the coefficient ($k_v$) describing linear concentration dependence of the sedimentation coefficient,
$s = s^0(1 - k_c c)$. In that regard we note that Batchelor’s value of 6.35$\phi$ for $k_h$ has since been decreased to 5$\phi$ by Brady and Dur- lowsky$^{27}$ – an amendment that is incorporated into subsequent considerations.

Upon noting that $2BM = 8\eta_c$ for an uncharged solute, the numerators of eqn (5) and (11) are equivalent for an isoelectric protein – as are the denominators in that Batchelor$^4$ has merely made the Stokes–Einstein substitution for $f^0$. Whereas $f^0$ was regarded as a constant in order to obtain eqn (7) as the quantitative expression for $k_h$, it is actually the frictional coefficient at zero protein concentration in a medium with viscosity $\eta$. Inasmuch as $\eta$ is a function of solute concentration, $f^0$ becomes a concentration-dependent variable of which account needs to be taken in the quantitative expression for $D$–$c$ dependence. As noted by Rowe,$^{37}$ the Batchelor treatments of migration$^{4,18-19}$ take no account of the solute contribution to viscosity of the medium.

An effect of viscosity on the magnitude of the translational diffusion coefficient is thus manifested in eqn (11), which we now write (with $k_s$ taken as 5$\nu_c$) in the form

$$D = \frac{(1 + 3\nu_c)RT}{6\pi c(\eta/\eta_b)\eta_c a}$$

(12)

where inclusion of the term for the ratio of solution to buffer viscosities, $\eta/\eta_b$, allows separation of this effect from that of buffer viscosity $[\eta_b]$, which is routinely eliminated by expressing $D$ as the value applying to diffusion in a medium with the viscosity of water at the temperature of interest.

Although $a$ is a hydrodynamic parameter, its magnitude is frequently taken as that of its thermodynamic counterpart [$a$ in eqn (8a)] in sedimentation equilibrium studies of interacting systems,$^{13,14,40,41}$ a practice for which there is supporting experimental evidence in the few instances where thermodynamic and hydrodynamic estimates of protein radius have been compared.$^{42}$ Such assumed identity of the Stokes (hydrodynamic) radius and the effective thermodynamic (excluded volume) radius is also supported by recent calculations based on coarse-grained models (4 beads per amino acid residue) for a range of proteins with widely differing sizes, shapes and non-uniform charge distribution.$^{43}$ Their assumed identity is also seemingly reasonable in that both parameters define the effective size of the protein molecule, especially when account is taken of the fact that sedimentation equilibrium reflects a balance between the two hydrodynamic processes of sedimentation and diffusion. On the grounds that the thermodynamic nonideality for a non-associating protein finds rational explanation in terms of physical interaction between solute particles rather than a change in radius,$^{4,14}$ we shall regard $a$ as a constant in eqn (12).

To take into account the concentration dependence of viscosity we introduce the expressions$^{44}$

$$\eta/\eta_b = 1 + \eta_{sp} c = 1 + [\eta] c + k_{sp} c^2$$

(13)

where the quadratic term in $c$ reflects concentration dependence of specific viscosity $\eta_{sp}$, the intrinsic viscosity ($[\eta]$) being the limiting value of $\eta_{sp}$ as $c \rightarrow 0$. For a spherical solute the intrinsic viscosity is readily obtained as the product $2.5\nu_s$, whereupon the expression for the concentration dependence of the diffusion coefficient becomes

$$D = \frac{(1 + 3\nu_c)RT}{N_A 6\pi c(1 + 2.5\nu_c c + \ldots)} \approx D^0(1 + 0.5\nu_c c)$$

(14)

for the concentration dependence of diffusion coefficient correct to first order in protein concentration. It should be noted that $D^0$ is indeed the diffusion coefficient in the limit of zero protein concentration in that $f^0$ now refers to the frictional coefficient at zero protein concentration in a medium with the buffer viscosity $\eta_b$. A much smaller concentration dependence of $D$ is now predicted.

**Consideration of experimental diffusion studies**

In view of the ambiguity surrounding the quantitative description of the concentration dependence of the diffusion coefficient, this aspect of experimental studies clearly needs to be examined. For that purpose we need experimental data obtained under the constraints of constant temperature and solvent chemical potential in order to comply with expression of the solute chemical potential gradient in terms of osmotic pressure [eqn (2) and (3)]. Unfortunately, that requirement excludes consideration of diffusion coefficients obtained by dynamic light scattering (photon correlation spectroscopy), for which the pertinent solute chemical potential is either $\mu_{T,P}$ or $\mu_{T,V}$. We must therefore go back half a century to the classical free diffusion experiments involving the spreading of an initially sharp boundary between protein solution and the diffusate against which it had been dialyzed extensively.$^{7,10,45}$ Furthermore, this was an era when the accuracy of diffusion coefficient measurement reached its peak in that several hours were spent generating the initial sharp boundary, after which diffusion was allowed to proceed for several days to generate concentration distributions with up to 5 cm between the solvent and solution plateaux. Indeed, the definition of the concentration distributions is sufficiently precise to allow delineation of the concentration dependence of $D$ from the extent of boundary asymmetry.$^{9}$

**Experimental studies of isoelectric ovalbumin**

Results$^{10}$ obtained from Rayleigh interference records of boundary spreading for ovalbumin in a Tiselius electrophoresis cell$^{46}$ are presented in Fig. 1. Most of the diffusion coefficients (●) refer to ovalbumin under isoelectric conditions ($pH$ 4.59, $I$ 0.16), but similar results (○) have also been obtained$^{45}$ under conditions ($pH$ 7.5 and 8.5, $I$ 0.10) where the protein bears a net charge of $-14$. Because the diffusion coefficients refer to the mean concentration across the boundary,$^{48}$ the abscissa of Fig. 1 is expressed in terms of the mean protein concentration $\tilde{c}$ rather than that ($2c$) in the solution plateau. Substitution of a value of 2.9 nm for $a$ (ref. 47, 49 and 50) into eqn (12) and (13) [with $f_j(x) = 0$ for $Z = 0$] gives an estimate of 246 L mol$^{-1}$ for $B_{2s}$, or $1.27 \times 10^{-4}$ mL mol$^{-1}$ for the usually reported second virial

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**Notes:**

1. Batchelor’s value of 6.35$\phi$ for $k_h$ has been decreased to 5$\phi$ by Brady and Durlowsky.
2. Stokes–Einstein substitution.
3. Concentration-dependent variable.
4. Thermodynamic and hydrodynamic estimates.
5. Coarse-grained models.
7. Sharp boundary.
8. Diffusion coefficient measurement.
10. Rayleigh interference.
11. Ovalbumin.
12. Tiselius electrophoresis cell.
13. Mean concentration.
coefficient \( (B) \) of this glycoprotein with a molecular mass of 44 kDa.\(^{23} \) Support for the use of \( 5p \) rather than \( 6.55p \) for \( k_s \) comes from a comparison of the respective estimates of 7.0 and 9.2 mL g\(^{-1} \) with the experimentally determined value of 7.6 mL g\(^{-1} \).\(^{23} \)

We are now in a position to compare the experimental results with the concentration dependence predicted by the three theoretical expressions [eqn (5), (12) and (14)]: because of the identity of \( 2BM \) and \( 8\nu \) for an uncharged solute, eqn (5) and (12) necessarily lead to the same predicted concentration dependence of \( D \). For non-spherical particles the covolume term, \( u_{\text{red}}v_e \), where the coefficient \( u_{\text{red}} \) (known as the reduced covolume) is 8 for spheres and greater than 8 for non-spherical particles, can be evaluated in terms of the axial dimensions of a particle based on theory for co-excluding triaxial ellipsoids under dominant Brownian motion\(^{25} \) – theory implemented in the algorithm COVOL.\(^{44} \) The experimental results have therefore been subjected to linear regression analysis according to eqn (6) with two assigned values of \( k_s \) to obtain \( D_0 \) and hence their best-fit description in terms of that model: \( k_D = +4.20 \text{ mL g}^{-1} \) for eqn (5) and (12); and \( k_D = +0.70 \text{ mL g}^{-1} \) for eqn (14), which incorporates the viscosity correction. For the first model the concentration dependence \( (\cdots) \), corresponding to the relationship \( 10^{-2} D = 3.90(1 + 4.20\epsilon) \), provides a poorer description of the experimental data than that \( (-----) \) emanating from the best-fit to eqn (14), \( 10^{-2} D = 3.93(1 + 0.70\epsilon) \). Linear least-squares analysis of the results in Fig. 1 to eqn (4) with \( D_0 \) and \( k_D \) as curve-fitting parameters yields a negative value \( (-2.0 \text{ mL g}^{-1}) \) for the concentration coefficient; but the uncertainty \( (\pm 2SD) \) of 2.6 mL g\(^{-1} \) exceeds its absolute magnitude. Inasmuch as the variation in diffusion coefficient predicted by eqn (14) is considerably smaller than the experimental uncertainty inherent in its measurement, the consideration of \( D \) to be a concentration-independent parameter for this system is justified.

As noted above, the results for an isoelectric protein do not distinguish between \( 8\nu \) and \( 2BM \) in the expressions for \( D-c \) dependence. However, the fact that similar estimates of \( D \) were obtained at neutral pH (where \( Z = -14 \) and hence \( 2BM \) is much larger) certainly implicates the covolume \( (8\nu) \) as the more appropriate parameter. This aspect is now examined further by considering the results from traditional diffusion studies of bovine serum albumin under conditions where the protein is decidedly anionic.

### Experimental studies of bovine serum albumin bearing net charge

An English-designed diffusimeter incorporating Gouy optics to record concentration gradient distributions\(^{35} \) was employed\(^{4} \) to obtain diffusion data for bovine serum albumin in phosphate buffer (pH 6.8, \( I = 0.10 \)), conditions under which the protein bears a net charge in the vicinity of \(-20.\)\(^{26} \) From Fig. 2 it is evident that the measured diffusion coefficients, taken from Table 4 of Creeth,\(^{4} \) exhibit no discernible concentration dependence; and the resultant average value of \( 6.14 (\pm 0.04) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1} \) for \( D_{29.8} \) indicates a Stokes radius of 3.5 nm. Incorporation of this value for \( a \) and \(-20 \) for \( Z \) into eqn (12) and (13) yields a molar second virial coefficient \( (B_{22}) \) of 765 000 mL mol\(^{-1} \), whereupon \( B = 1.76 \times 10^{-4} \text{ mL mol}^{-1} \text{ g}^{-2} \) for the bovine serum albumin, which has an analytical molecular mass of 66 kDa.\(^{57} \) In keeping with the observations for ovalbumin, the lower estimate of \( k_s \) (8.3 cf. 10.9 mL g\(^{-1} \)) is closer to the generally accepted range of 7–8 mL g\(^{-1} \) that is considered to describe this parameter for globular proteins.

Analysis of the experimental results in accordance with eqn (6) using the calculated value of \( k_D (+15.0 \text{ mL g}^{-1}) \) from eqn (5) to obtain \( D_0 \) and hence the best-fit theoretical description according to eqn (5) yields the expression \( 10^{-2} D = 5.93(1 + 15.0\epsilon) \), which provides a relatively poor fit \( (\cdots) \) of the data. The disparity between experiment and prediction is certainly decreased by employing eqn (12), a change which leads to the best-fit description \( 10^{-2} D = 6.07(1 + 4.92\epsilon) \) and the broken line \( (\cdots\cdots) \) in Fig. 2; and decreased still further \( (-----) \) by incorporating the viscosity correction to obtain the best-fit relationship \( 10^{-2} D = 6.13(1 + 0.82\epsilon) \). Linear least-squares analysis of the results in Fig. 2 to eqn (6) with \( D_0 \) and \( k_D \) as curve-fitting parameters yields a slightly negative value \( (-1.80 \text{ mL g}^{-1}) \) for the concentration coefficient; but the uncertainty \( (\pm 2.6 \text{ mL g}^{-1}) \) again exceeds its absolute magnitude. As with ovalbumin (Fig. 1), the variation in diffusion coefficient predicted by eqn (14) is within the experimental uncertainty of an experimental measurement, whereupon the consideration of \( D \) to be concentration-independent is an acceptable approximation for this system as well.

![Fig. 1](image1.png) Concentration dependence of the translational diffusion coefficient for ovalbumin under isoelectric conditions (•) and at neutral pH (○), together with theoretical relationships predicted by eqn (5) or eqn (12) (---), and eqn (14) (-----). [Data taken from Table 1 of Creeth\(^{4} \) and Table 1 of Nichol et al.\(^{49} \) respectively.]

![Fig. 2](image2.png) Concentration dependence of the translational and sedimentation coefficients for bovine serum albumin. (A) Experimental diffusion coefficients\(^{4} \) obtained at neutral pH (pH 6.8, \( I = 0.10 \)), together with the theoretical relationships predicted by eqn (5) (• • • • •), eqn (12) (-----), and eqn (14) (-----). [Experimental data taken from Table 4 of Creeth.\(^{4} \)]
An interesting point to emerge from this assessment of diffusion data for a charged protein system is the much better theoretical description that is obtained by regarding the covolume term, \( u_{\text{cov volumen}} \), rather than the excluded volume (2BM) as the parameter contributing to hydrodynamic nonideality. In other words, the nonideality of Brownian motion seems to be governed by the actual size of the spherical solute, whereas the covolume has to be supplemented by the contribution arising from considerations of the potential-of-mean-force between charged molecules [see eqn (12) and (13)] in order to describe thermodynamic nonideality. The consequent lack of an effect of charge on \( D \), which was noted for ovalbumin (Fig. 1), is also observed with bovine serum albumin in that the diffusion coefficients (\( D_{20,\text{w}} \)) of \( 6.02 \times 10^{-7} \) and \( 6.10 \times 10^{-7} \) cm\(^2\) s\(^{-1}\) inferred from traditional studies of essentially isoelectric protein\(^{46,59} \) are encompassed by the envelope of scatter in Fig. 2.

These findings of minimal dependence of diffusion coefficients on either the charge or concentration for both ovalbumin and bovine serum albumin are clearly at variance with conclusions drawn from studies of the extent of boundary spreading in sedimentation velocity experiments,\(^{46,59}\) for which a better quantitative description was obtained by assigning a magnitude to \( k_D \) in the above eqn (6) as the expression for \( D-c \) dependence – a reasonable course of action in view of the latest theoretical treatment of \( D-c \) dependence\(^{5} \) at the time. However, such observation of a decrease in the sum-of-squares-of-residuals\(^{17} \) merely establishes that better agreement between experiment and theory can be achieved by introducing an additional curve-fitting parameter to decrease slightly the predicted extent of boundary sharpening; and the present conclusions based on considerations of boundary spreading arising solely from a gradient in solute chemical potential render less likely the validity of identifying that additional curve-fitting parameter as \( k_p \). Although the only other recent sedimentation velocity investigation to take account of boundary sharpening resulting from \( s-c \) dependence\(^{48} \) also incorporated eqn (6) to allow for concentration dependence of the diffusion coefficient for pegaseys, such action may be vindicated by the attachment of a polymer chain (polyethylene glycol) to the pegaseys polypeptide chain, a feature which disqualifies its consideration as a globular protein – the only system for which the present claims about \( D-c \) independence have been substantiated.

**Discussion**

A major purpose of this investigation has been to show limitations of a theoretical treatment that has been developed for the concentration dependence of translational diffusion coefficients for globular proteins.\(^{5,6} \) Although seeming agreement between experiment and prediction was observed,\(^{45,62} \) those comparisons (on spherical plant viruses) entailed diffusion coefficients obtained by dynamic light scattering – a technique involving the measurement of \( D \) under the constraint of constant pressure rather than constant solvent chemical potential. Because the latter constraint is specified in the theoretical treatment by virtue of expressing the gradient in solute chemical potential in terms of the corresponding gradient in osmotic pressure [eqn (3)], diffusion studies of proteins under conditions that comply with the specified constraint (constant solvent chemical potential) have been sought in the current investigation. Whereas significant positive concentration dependence of the diffusion coefficient for both proteins is the theoretical prediction of eqn (5), none has been detected experimentally in traditional diffusion studies of either bovine serum albumin\(^{8} \) or ovalbumin.\(^{10,45} \) Instead, the results tend to favor description in terms of the relatively minor concentration dependence predicted by eqn (14), which substitutes the covolume (8\( v_s \)) for the thermodynamic second virial coefficient term (2BM) in eqn (5) and also includes a viscosity correction term. In that regard we note that such interpretation is also supported by the report\(^{9} \) of only slight negative concentration dependence \( (k_D = -1.0 \) cm\(^3\) g\(^{-1}\) \) based on interpretation of measurements of boundary skewness measurements for serum albumin. Indeed, essentially the same concentration coefficient \( (k_D = -1.1 \) cm\(^3\) g\(^{-1}\) \) also applies to the diffusion of \( \beta \)-lactoglobulin.\(^{7,9} \) On the grounds that these predicted variations in \( D \) for plateau concentrations in the range 1–10 mg mL\(^{-1}\) (0.5 \( \leq \zeta \leq 5 \)) are also within the uncertainty of experimental estimates, they provide additional justification for neglecting \( D-c \) dependence in analyses of boundary spreading by globular proteins in sedimentation velocity studies, where the constraints of constant temperature and solvent chemical potential also seem to apply.\(^{27-29} \) On the other hand, reports of significant positive concentration dependence of \( D^{11,62} \) have emanated from dynamic light scattering measurements, where the constraint of constant pressure rather than constant solvent chemical potential applies to the solute chemical potential being monitored. Because the latter constraint is specified in the theoretical treatment by virtue of expressing the gradient in solute chemical potential in terms of the corresponding gradient in osmotic pressure [eqn (3)], diffusion studies of proteins under conditions that comply with the specified constraint (constant solvent chemical potential) have been sought in the current investigation. Whereas significant positive concentration dependence of the diffusion coefficient for both proteins is the theoretical prediction of the current expression for \( D-c \) dependence [eqn (5)], none has been detected experimentally in traditional diffusion studies of either bovine serum albumin\(^{9} \) or ovalbumin.\(^{10,45} \) As noted above, the results tend to favor description in terms of the relatively minor concentration dependence predicted by eqn (14), which substitutes the covolume (8\( v_s \)) for the thermodynamic second virial coefficient term (2BM) in eqn (5) and also includes a correction term for solute viscosity. In that regard we note such action is counter to that adopted in dynamic light scattering studies, where buffer viscosity is regarded as the appropriate parameter.\(^{15-16} \) However, because of the constraint of constant pressure that applies in those experiments, the buffer constituents must be regarded as additional solutes\(^{17-19} \) whose contribution would dominate the solution viscosity relative to that of water (the solvent). In diffusion studies under the constraint of constant solvent chemical potential the buffer constituents become part of the solvent, whereupon the only contributor to the relative viscosity \( (\eta/\eta_s) \) is the protein solute.
Another factor that could also decrease the $D-c$ dependence from that predicted by the Batchelor expression \[ \text{[eqn (10) as modified by Brady and Durlofsky]} \] is the operation of solute–solvent interaction arising from the coupling of ion flows.\(^6\) Indeed, for a protein bearing net charge the condition of constant solvent chemical potential generates a reverse concentration gradient in buffer/electrolyte ions to accommodate the Donnan effect.\(^6\) On the grounds that the magnitude of this reverse concentration gradient in buffer and electrolyte ions becomes greater with increasing protein concentration, the mediation of overall protein diffusion by such coupling of ion flows could also be partly responsible for the concentration independence of $D$ that is observed experimentally.

Inasmuch as the current interest in quantifying the concentration dependence of $D$ resides in analysis of boundary spreading in sedimentation velocity studies of proteins,\(^10\)\(^{16}\) we again draw attention to the fact that an approximate analytical solution of the Lamm equation\(^4\) already exists\(^5\) for the situation encountered above in which $D$ is essentially concentration independent and $s$ exhibits linear concentration dependence: this study provides a sound theoretical basis for reintroducing such an analysis. Alternatively, it provides the basis for simplifying current simulative procedures\(^10\)\(^{16}\) by the elimination of $k_B$, the coefficient describing the $D-c$ dependence for globular proteins \[ \text{[eqn (6)]}, \] as an additional curve-fitting parameter to be evaluated from the analysis.

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**Notes and references**