COVOL: An Interactive Program for Evaluating Second Virial Coefficients from the Triaxial Shape or Dimensions of Rigid Macromolecules

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ABSTRACT An interactive program is described for calculating the second virial coefficient contribution to the thermodynamic nonideality of solutions of rigid macromolecules based on their triaxial dimensions. The FORTRAN-77 program, available in precompiled form for the PC, is based on theory for the covolume of triaxial ellipsoid particles [Rallison, J. M., and S. E. Harding. (1985). *J. Colloid Interface Sci*. 103:284–289]. This covolume has the potential to provide a magnitude for the second virial coefficient of macromolecules bearing no net charge. Allowance for a charge–charge contribution is made via an expression based on Debye–Hückel theory and uniform distribution of the net charge over the surface of a sphere with dimensions governed by the Stokes radius of the macromolecule. Ovalbumin, ribonuclease A, and hemoglobin are used as model systems to illustrate application of the *COVOL* routine.

INTRODUCTION

The interpretation of thermodynamic equilibrium data such as those derived from sedimentation equilibrium distributions in the analytical ultracentrifuge, as well as those from classical (static) light scattering and osmotic pressure measurements for biological and other macromolecules in terms of the molecular weight or the stoichiometry and strength of interactions between macrolecules, is often influenced by contributions from the thermodynamic nonideality of the system. This nonideality, which exists at all finite concentrations, derives from two sources (see, e.g., Tanford, 1961): an excluded volume (covolume) contribution emanating from the large size of macromolecules relative to that of solvent molecules; and, in aqueous systems, a polyelectrolyte contribution deriving from the net charge (valence) of many macromolecular species—particularly those of biological origin (proteins, nucleic acids, polysaccharides, and glycoconjugates). For some macromolecules at high dilution, such contributions are sufficiently small to warrant their neglect. Alternatively, measurements at a series of concentrations may be extrapolated to zero concentration to eliminate effects of nonideality (Tanford, 1961). Unfortunately, both of those procedures tend to compromise the analysis of properties that are concentration dependent; in particular, the study of interactions between macromolecules—an area that underpins the whole of biological science (Schachman, 1989).

From the quantitative expression for the polyelectrolyte contribution to thermodynamic nonideality for spherical macromolecular solutes (Wills et al., 1980; Winzor and Wills, 1995), it is evident that the extent of nonideality stemming from this source may be decreased either by increasing the ionic strength of the solvent or, in the case of proteins, by selecting a pH in the vicinity of the isoelectric point. In contrast, the covolume contribution is independent of solvent conditions. Covolume formulations are available for certain types of centrosymmetric rigid structures. Of these, the simplest is the sphere, but expressions have also been derived for ellipsoids of revolution (Isihara, 1950; Ogston and Winzor, 1975) and for the triaxial ellipsoid in which all three semiaxes differ in magnitude (Rallison and Harding, 1985). This triaxial ellipsoid with semiaxial dimensions $a \geq b \geq c$ (Fig. 1) clearly provides the most general example of a rigid centrosymmetric particle. Although complicated structures, such as an immunoglobulin or complement system, are not accurately described, rod-shaped ($a \gg b \approx c$), disc-shaped ($a \approx b \gg c$), globular shaped ($a \geq b \geq c$), and even tape-shaped ($a \gg b \gg c$) macromolecules can all be represented adequately by such means. Indeed, because the required covolume is a time-averaged parameter for macromolecules under dominant Brownian motion, the representation of even an immunoglobulin in terms of one of the above shapes will almost certainly suffice for description of thermodynamic nonideality effects on the magnitude of an equilibrium thermodynamic property. However, for such irregular shaped macromolecules, a recent development has been to extend, to the case of covolume calculations, multiple-sphere or bead-modeling approaches for a structure that, although approximate in terms of its hydrodynamics and thermodynamics, can give better representations of structure. For covolume, a Monte Carlo procedure has been incorporated into the most recent version of the general bead-modeling algorithm *SOLPRO* (Garcia de la Torre et al., 1999), by sampling or "trialing" all possible orientations of two-particle interac-

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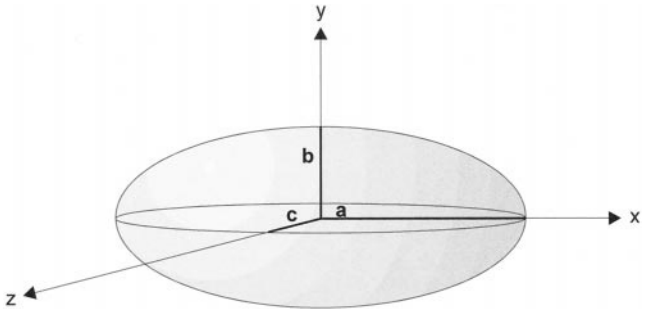

FIGURE 1 Schematic representation of a rigid macromolecule as a triaxial ellipsoid in which all three semiaxes (a, b, c) can differ in length. Its shape is characterized by the two axial ratios (a/b, b/c)

tions and checking for overlap of any bead in one molecule with any in its neighbor. The precision of this method obviously increases with increase in the number of trials. With a moderate number of trials taking, for example, 50 min in a Pentium 200 computer, estimates for the covolume can be returned to a precision of better than ~10%. However, for more regular structures, the exact covolume relations for triaxial ellipsoids are more useful. Both the bead and the general ellipsoidal deliberations are based on the premise that solutions are sufficiently dilute to allow the consideration of thermodynamic nonideality solely in terms of two-particle interactions, whereupon effects of thermodynamic nonideality become manifested in the magnitude of the second virial coefficient.

At present, considerable interest is centered on the use of scaled particle theory to analyze the thermodynamic activity of concentrated protein solutions in terms of a single parameter—the effective radius of the hard particle (Ross and Minton, 1979; Berg, 1990; Guttman et al., 1995). An obvious attribute of this approach is its ability to extend the analysis of experimental data beyond the concentration range for which description of nonideality in terms of a second virial coefficient ceases to be a valid approximation. However, the lack of any specific allowance for the charge–charge contribution to thermodynamic nonideality means that the quantitative description in such terms only applies to the system under the conditions (pH, ionic strength) of the experiment subjected to analysis. Conclusions about nonideality effects in solutions of the same protein at (say) a different ionic strength are precluded because the change in the charge–charge contribution necessitates redetermination of the empirical-scaled particle parameter (effective radius) that is required to describe the nonideality under the new condition. We therefore retain the classical statistical-mechanical approach.

The triaxial ellipsoid expressions (Rallison and Harding, 1985) were devised with the intention of combining hydrodynamic parameters with measurements of the second virial coefficient to estimate macromolecular shape in solution (Harding, 1989). However, a greater potential now seems to be the use of macromolecular structure details to predict the magnitude of thermodynamic activity coefficients that are required to make allowance for nonideality effects in the evaluation of equilibrium constants for macromolecular interactions (Winzor and Wills, 1995; Wills et al., 1996). *COVOL* has been developed with this objective in mind.

THE SECOND THERMODYNAMIC VIRIAL COEFFICIENT

The simplest way to represent thermodynamic nonideality of macromolecular solutions, correct to first order in concentration, is in terms of the second virial coefficient B (sometimes designated as A_2). It may be envisaged as a measure of the extent to which a determined value of the apparent molar mass M_{app}, at finite concentration c, underestimates the true parameter M. For molecular weight measurement by osmotic pressure, the relationship is (Tanford, 1961)

$$1/(M_n)_{app} = 1/M_n + Bc + \cdots, \quad (1)$$

where the additional subscript (n) signifies that the number-average molecular weight is measured by osmometry. For measurements of molecular weight from either absorption or Rayleigh interference records of sedimentation equilibrium distributions in the analytical ultracentrifuge, and also from classical (static) light scattering data, the corresponding expression is

$$1/(M_w)_{app} = 1/M_w + 2Bc + \cdots, \quad (2)$$

where the w subscript denotes the weight-average nature of the molecular weight determined by these methods. Use of the qualifying coefficient in the concentration term of Equ. 2 allows retention of the osmotic virial coefficient B for the description of nonideality in the various experimental methods of molecular weight measurement.

EXCLUDED VOLUME CONTRIBUTION, B_{ex}

The excluded volume (or covolume) of a macromolecule, u, is the volume of solution (frequently expressed in mL) from which the centers of two molecules are mutually excluded. For the simple situation of an impenetrable spherical particle with radius r, the distance of closest approach is $2r$, in which case $u = \frac{4}{3}\pi(2r)^3 = 8V$, where V is the volume of the particle. To obtain a normalized parameter related solely to shape, Rallison and Harding (1985) introduced the concept of a reduced covolume, u_{red}, defined as the excluded volume per unit particle volume. The excluded volume then becomes the product of the shape parameter, u_{red} (with a minimal value of 8), and the particle volume, which takes into account the degree of swelling of the macromolecule through solvation ($u = Vu_{red}$). By expressing V in terms of the specific solvated volume v_s, i.e., the volume of the solvated particle per unit unsolvated mass, the relationship between excluded volume and reduced excluded volume

may be written as

$$u = (v_s M/N_A)u_{red}, \tag{3a}$$

where the ratio of molar mass (M) to Avogadro's number (N_A) has been substituted for the molecular mass. This is equivalent to the approach (Tanford, 1961) in which v_s is regarded as the sum of the unsolvated partial specific volume $\bar{v}$ and a term for particle solvation. So, $v_s = \bar{v} + \delta/\rho_o$, where ρ_o is the solvent density and δ the extent of solute solvation (g solvent per g solute). Because of the greater popularity of this approach, Eq. 3a is usually written in the form

$$u = \{[\bar{v} + (\delta/\rho_o)]M/N_A\}u_{red}. \tag{3b}$$

In the fields of colloid and polymer chemistry, the virial expansion is traditionally defined with c expressed in g/mL, whereupon the dimensions of the second virial coefficient B become mL mol g^{-2}. In these terms, the excluded (or covolume) contribution to the second virial coefficient, B_{ex}, is given by the relationship

$$B_{ex} = uN_A/(2M^2). \tag{4}$$

POLYELECTROLYTE CONTRIBUTION, B_Z

In studies of charged macromolecules such as proteins and polyelectrolytes in aqueous solution, the effective distance of closest approach is greater than that based on geometrical considerations because of the repulsive force opposing the approach of two particles bearing net charge (valence) Z. This additional contribution to the second virial coefficient, B_Z, has only been evaluated explicitly for impenetrable spheres. For such systems, the expression for the second virial coefficient, B, is given by (Wills et al., 1980; Winzor and Wills, 1995)

$$B = B_{ex} + B_z = \frac{uN_A}{2M^2} + \frac{1000Z^2}{4M^2I}\left(\frac{1 + 2\kappa r_s}{(1 + \kappa r_s)^2} + \cdots\right), \tag{5}$$

where the factor of 1000 is introduced to accommodate the conventional definition of ionic strength I (mol/L), κr_s is the product of the inverse screening length (Debye and Hückel, 1923) and the solvated radius, r_s, of the particle. The Stokes radius provides an acceptable estimate of r_s (cm), irrespective of macromolecular shape, and the magnitude of κ (cm^{-1}) may be evaluated from the expression $\kappa = 3.27 \times 10^7\sqrt{I}$ at 20°C.

CALCULATION OF B_{ex} FROM THE TRIAXIAL DIMENSIONS OF A RIGID IMPENETRABLE MACROMOLECULE

As noted above, the simplest situation for which u_{red} is known is a sphere, where $u_{red} = 8$. This is the minimal value for u_{red} of a triaxial ellipsoid, for which the general expression is

$$u_{red} = 2 + [3/(2\pi abc)]SR, \tag{6}$$

where S and R are the double-integral functions defined in Eqs. 3 and 4 of Rallison and Harding (1985). Although it is possible to solve analytically one of the double integrals in each of the expressions for S and R, the results are sufficiently complicated that it is easier to perform all of the integrals by numerical integration.

SECOND VIRIAL COEFFICIENTS DEFINED ON A MOLAR BASIS

From the viewpoint of allowing for effects of thermodynamic nonideality in the characterization of macromolecular equilibria, there is merit in defining the second virial coefficient on a molar rather than a molecular basis. In terms of molar covolume, $U = uN_A$, Eq. 3 becomes

$$U = (v_s M)u_{red} = [(\bar{v} + \delta/\rho_o)]Mu_{red}. \tag{7}$$

U, in turn, is related to the second virial coefficient defined in molecular terms, B_{ex}, by the relation (Tanford, 1961; Ogston and Winzor, 1975; Jeffrey et al., 1977)

$$B_{ex} = U/(2M^2). \tag{8}$$

COVOL

COVOL, an interactive FORTRAN 77 algorithm written for PC, evaluates B_{ex} by enumerating S and R, and hence u_{red}, from user-specified values of the three semiaxes a, b, and c (or, alternatively, a/b and b/c because of the sole dependence of u_{red} upon shape), through Eq. 6. The double integrals S and R of Eq. 6 are evaluated using the Numerical Algorithms Group (1992) numerical integration routine D01DAF.

The next stage is the evaluation of the molar covolume U from u_{red} and user-specified values for the molecular weight (M) and either the solvated specific volume (v_s) (Eq. 3a) or the unsolvated partial specific volume (v), the solvation (δ), and the solvent density (ρ_o), through Eq. 3b. The routine prints out the molar excluded volume, U, the molecular excluded volume, u ($= U/N_A$), and B_{ex} (Eq. 4). At that stage the program asks whether there is an additional contribution to B from polyelectrolyte behavior. If yes, the user enters the ionic strength (mol/L) and net charge (valence) of the macroion, Z. After evaluation of B according to Eq. 5, the routine concludes by printing out the charge–charge contribution (B_z) and the magnitude of the second virial coefficient, $B = B_{ex} + B_z$.

A flow chart for the program is given in Fig. 2. The FORTRAN 77 compiler, Salford FTN77/486 system (Salford, 1991) and the Numerical Algorithms Group (1991) numerical integration routine D01DAF are built into the program; no separate FORTRAN or NAG compilers are required. *COVOL* is available in either precompiled or

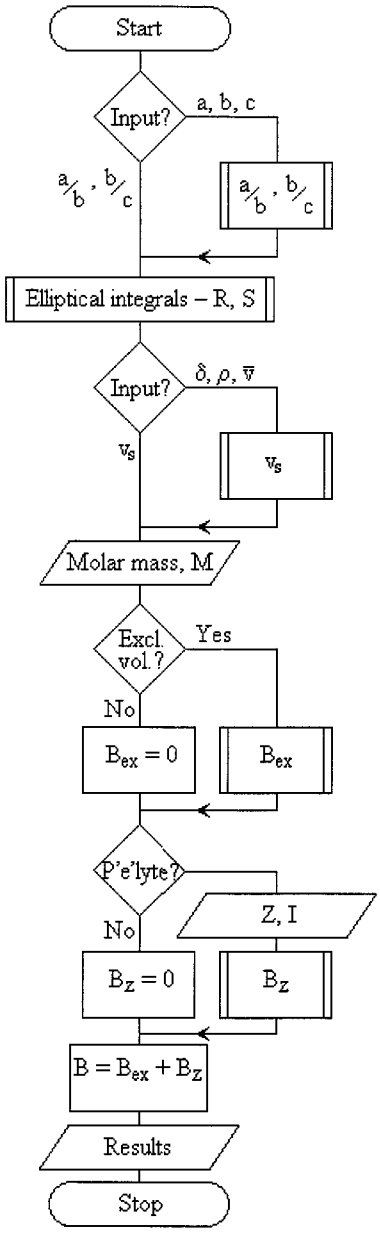

FIGURE 2 Flow chart of the *COVOL* routine for calculating second virial coefficients from the triaxial ellipsoid shape or dimensions and net charge (valence) of a rigid macromolecule.

source-code form from Steve.Harding@nottingham.ac.uk or from the web page http://www.nottingham.ac.uk/ncmh/.

USER INPUT OF AXIAL RATIOS BASED ON CRYSTALLOGRAPHIC COORDINATES OF A MACROMOLECULE

An objective method for defining the triaxial shape of a protein molecule from its atomic structural coordinates has been provided by Taylor et al. (1983). This method, which is insensitive to small deviations from ideal ellipsoidal form, is based on the inertial, momental, or Cauchy ellipsoid dilated so that it forms a close approximation to the protein surface. The procedure is in the form of a FORTRAN 77 algorithm called *ELLIPSE*. A recent version of the algorithm, implemented by Hubbard (1994), was used to calculate the ratios of the principal axes of the equimomental ellipsoid for the three-dimensional coordinates of a protein. These ratios can be used in conjunction with a second algorithm, *SURFNET*, (Laskowski, 1995) to generate a three-dimensional surface representation of the ellipsoid (Fig. 3). *SURFNET* can be downloaded from page http://www.biochem.ucl.ac.uk/~roman/surfnet/surfnet.html.

APPLICATION OF *COVOL*

The use of *COVOL* for prediction of the magnitude of second virial coefficients is explored first by consideration of ovalbumin, a protein whose high resolution crystal structure was recently published (Stein et al., 1991). Fitting the crystal coordinates to the inertial ellipsoid using *ELLIPSE* yielded axial ratios (a/b, b/c) of (1.87, 1.08). The resulting fit is shown in Fig. 3. Input of these respective values for a/b, and b/c into *COVOL* yields a reduced covolume, u_{red}, of 8.996. Conversion of this reduced covolume to a covolume, B_{ex}, depends upon the magnitude assigned to the solvation parameter (δ) for this protein with a partial specific volume ($\bar{v}$) of 0.748 mL/g (Dayhoff et al., 1952) and a molecular weight of 45,000 (Jeffrey et al., 1977). The effect of the extent of solvation upon the magnitude of B_{ex} calculated by Eqs. 3b and 4 is summarized by the solid line in Fig. 4, where the intersecting horizontal dashed lines denote the estimates of B deduced experimentally from sedimentation equilibrium (Jeffrey et al., 1977) and size exclusion chromatography studies (Shearwin and Winzor, 1990) of isoelectric ovalbumin (*upper* and *lower lines*, respectively). It is noted that the consequent estimates of 0.49 ($\pm$ 0.05) and 0.39 ($\pm$ 0.18) for the extent of ovalbumin solvation δ are at the upper end of, or greater than, the usually accepted range (0.3–0.4) for globular proteins (Oncley, 1941; Tanford, 1961; Zhou, 1995). Experimental support for a higher value is provided by concordance of estimates (2.92 nm) for the Stokes radius and the effective radius deduced from the molar covolume, $U = {}^{32}\!/_{3}\pi N_A r^3$. A similar conclusion about the extent of solvation stems from size-exclusion chromatography studies (Shearwin and Winzor, 1990) in phosphate-chloride buffer, pH 7.4, I 0.156, conditions under which a net charge (Z) of -16 results in a polyelectrolyte contribution to B (Eq. 5). The upper dependence (*dash-dot line* of Fig. 4) summarizes the calculated variation of B with δ, whereas the intersecting horizontal line denotes the experimental value of B obtained by exclusion chromatography. On this basis, ovalbumin is hydrated to the extent of 0.42 ($\pm$ 0.09).

Analysis of the dependences of the second virial coefficient upon extent of solvation for isoelectric ribonuclease A and hemoglobin are presented in Fig. 5. For ribonuclease A, the atom coordinates stored in the x-ray crystallographic database (Borkakoti et al., 1984) signify semiaxial ratios of

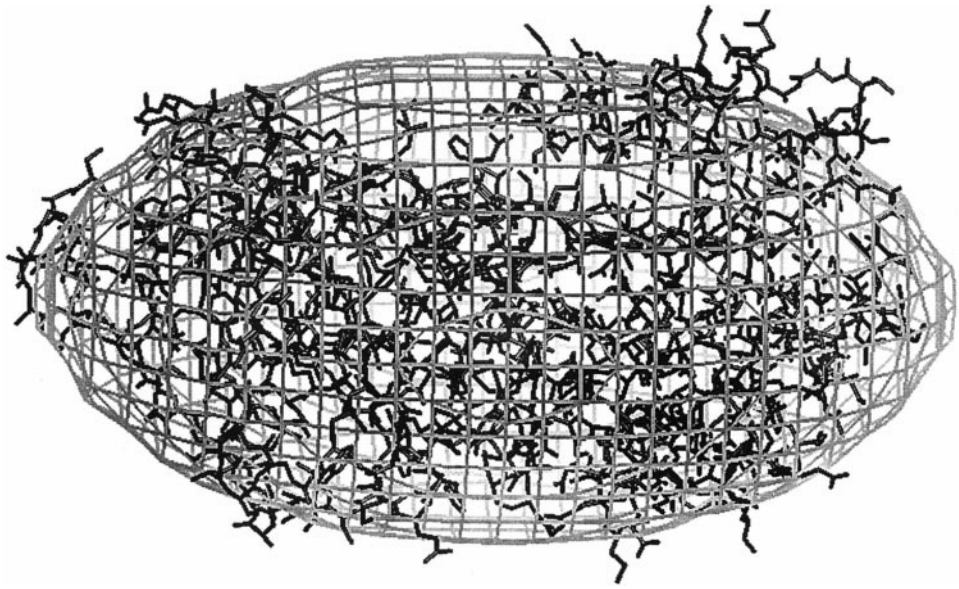

FIGURE 3 Inertial ellipsoid fitted to the crystal structure for ovalbumin (Stein et al. 1991). The axial ratios for the ellipsoid were calulated using *ELLIPSE* and the surface diagram was generated using *SURFNET* (Laskowski, 1995).

1.53 and 1.23, which yield a reduced covolume, u_{red}, of 8.692 for this enzyme, with $M = 13{,}700$ and $\bar{v} = 0.703$ mL/g. The atom coordinates of human deoxyhemoglobin (Fermi et al., 1984) give rise to semiaxial ratios of 1.27 and 1.07, and hence to a reduced covolume of 8.170 for this protein, with $M = 64{,}500$ and $\bar{v} = 0.746$ mL/g. On the basis of the horizontal broken lines, which correspond to experimentally determined values of B for ribonuclease A (Shearwin and Winzor, 1990) and hemoglobin (Baghurst et al., 1974), the respective extents of hydration are 0.25 and 0.43 g/g. In the latter regard, we note that values of 0.35–0.54 g/g have been reported by Guttman et al. (1995) by analysis of the thermodynamic nonideality of concentrated hemoglobin solutions in terms of the Berg (1990) adaptation of scaled particle theory (Ross and Minton, 1979).

An obvious difficulty with calculation of the second virial coefficient by this means is the pronounced dependence of

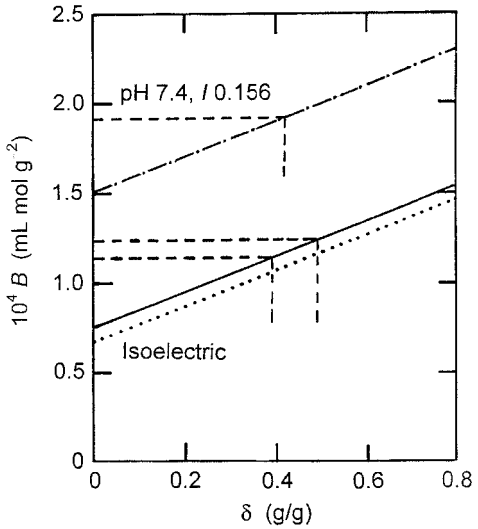

FIGURE 4 Effect of the extent of solvation (δ) upon the magnitude of the second virial coefficient (B) calculated by *COVOL* on the basis of ratios of triaxial ellipsoid semiaxes of 1.87 (a/b) and 1.08 (b/c) for isoelectric ovalbumin (— —), and for the same protein under conditions (pH 7.4, I 0.156) where it bears a net charge (valence) of −16 (–·–·–). · · · · · ·, corresponding dependence for isoelectric ovalbumin modeled as a sphere. *Horizontal lines* denote experimental estimates of B from sedimentation equilibrium and exclusion chromatography studies of ovalbumin.

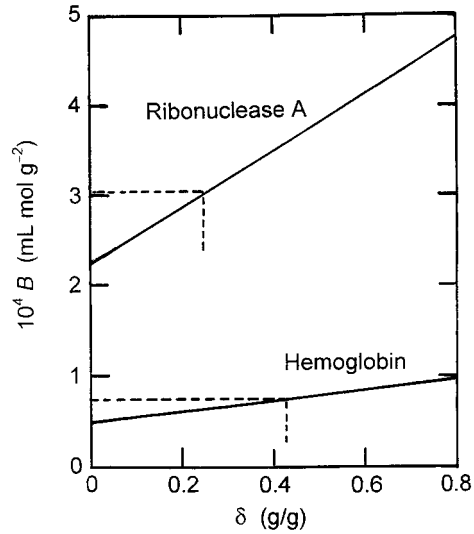

FIGURE 5 Effect of the extent of solvation (δ) upon the magnitude of the second virial coefficient (B) on the basis of the ratios of triaxial ellipsoid semiaxes for isoelectric ribonuclease and hemoglobin. *Horizontal broken lines* denote experimental estimates of B obtained from exclusion chromatography studies of the enzyme and from osmotic pressure measurements for hemoglobin.

B_{ex} upon the magnitude assigned to δ, a parameter for which the value is often very subjective because it has rarely been determined. Indeed, this sensitivity of B_{ex} to the value of δ relegates to secondary importance the relative magnitudes of the triaxial ellipsoid semiaxes—a factor evident from the dotted dependence in Fig. 4, which refers to isoelectric ovalbumin modeled as a sphere (u_{red} = 8.000). Values of 0.47–0.57 for the extent of ovalbumin hydration are obtained from this model and the exclusion chromatographic (Shearwin and Winzor, 1990) and sedimentation equilibrium (Jeffrey et al., 1977) estimates of B.

ALLOWANCE FOR NONIDEALITY IN SOLUTE SELF-ASSOCIATION

Thus far, the investigation has been dominated by considerations of the procedure for predicting magnitudes of the second virial coefficient—on the grounds that values need to be assigned to these parameters to account for the effects of thermodynamic nonideality in the quantitative characterization of macromolecular interactions. We illustrate such use of second virial coefficients (defined initially on the molar basis) by considering the situation for a reversibly dimerizing solute.

For a monomer ⇌ dimer system the molar thermodynamic activity of monomer, z_1, which differs from its molal counterpart (a_1) because of the different constraints entailed in the definitions of the chemical potential of solute (Winzor and Wills, 1995), is related to the base-molar solute concentration C (weight-concentration divided by monomer molecular weight M_1) by the expression

$$C = z_1 + 2(K_2 - B_{11})z_1^2 + \cdots, \qquad (9)$$

where K_2 is the dimerization constant (L/mol) and B_{11} is the molar second virial coefficient reflecting monomer–monomer excluded volume interactions (Wills et al., 1996, 1997). Interpretation of the quadratic coefficient of the dependence of total solute concentration upon monomer activity in terms of the dimerization constant K_2 is thus predicated upon specification of a value for B_{11}. Statistical-mechanical considerations establish the relationship,

$$B_{11} = (U_{11}/2) + (Z^2/4I)[(1 + 2\kappa r_1)/(1 + \kappa r_1)^2], \qquad (10)$$

where r_1 is the effective monomer radius and U_{11} is the molar monomer–monomer covolume. On noting that $K_2 = X_2M_1/2$ is the relationship between dimerization constants defined on molar (K_2) and weight (X_2) bases, Eq. 9 may also be written in terms of B for monomer (Eq. 6) as

$$c = M_1z_1 + (X_2 - 2BM_1)(M_1z_1)^2 + \cdots, \qquad (11)$$

for the treatment of data analyzed in terms of total weight concentration c.

CONCLUDING REMARKS

This investigation has demonstrated the use of *COVOL* to calculate second virial coefficients for macromolecules that can be modeled as impenetrable triaxial ellipsoids; but has also identified the problem that realization of its full potential must await more definitive means of assessing the magnitude of δ, the extent of macromolecule solvation. In that regard, the extent of solvation has usually been considered to be in the range 0.3 to 0.4 for globular proteins (Oncley, 1941; Tanford, 1961), whereas experimental measurements of B for ovalbumin signify a higher value (0.4 to 0.6) for δ. The value of 0.25 for ribonuclease is marginally below the considered range. Measurements of B for a range of proteins with known axial dimensions are clearly required to shed further light on the likely magnitude of δ and, hence, on its prediction on the geometrical basis of an assigned thickness to the solvation layer extending over the surface of the protein molecules (see, e.g., Jacobsen et al., 1996). It is, therefore, hoped that this investigation may stimulate renewed interest in accurate measurement of osmotic virial coefficients—parameters for which the major use in the past has merely been to guide the elimination of nonideality by extrapolation of data to infinite dilution.

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