Free-Solvent Model of Osmotic Pressure Revisited. Application to Concentrated IgG Solution at Physiological Conditions

ARTICLE *in* JOURNAL OF COLLOID AND INTERFACE SCIENCE · FEBRUARY 1998

Impact Factor: 3.37 · DOI: 10.1006/jcis.1997.5262 · Source: PubMed

3 AUTHORS:

Mohammed Yousef
Bio-Rad Laboratories
14 PUBLICATIONS **227** CITATIONS
SEE PROFILE

Ravindra Datta
Worcester Polytechnic Institute
161 PUBLICATIONS **3,507** CITATIONS
SEE PROFILE

Victor G J Rodgers
University of California, Riverside
65 PUBLICATIONS **919** CITATIONS
SEE PROFILE

JOURNAL OF COLLOID AND INTERFACE SCIENCE **197,** 108–118 (1998)
ARTICLE NO. CS975262

Free-Solvent Model of Osmotic Pressure Revisited: Application to Concentrated IgG Solution under Physiological Conditions

M. A. Yousef,* R. Datta,* and V. G. J. Rodgers*,†,[1]

*The Department of Chemical and Biochemical Engineering and †Center for Biocatalysis and Bioprocessing, The University of Iowa, Iowa City, Iowa 52242

Received May 15, 1997; accepted October 24, 1997

The osmotic pressure measurements of bovine immuno-gamma globulin in phosphate-buffered solution at pH 7.4 and 0.13 M total salt concentration were extended to near saturation concentrations for ambient temperature. The osmotic pressure at the highest measured concentration of 424 g/L was 4.18 psi (28.3 kPa). A free-solvent model, considering solute–solvent interaction in the concentration variable, provided an excellent fit to observed osmotic pressure nonideality at even the highest protein concentration. The calculated mass of hydrated solvent compared with amounts determined from water–^{17}O magnetic resonance for other globular proteins. This model provides an improved correlation to the data over virial equations (truncated to the third term) when only solute–solute interactions are considered. The use of mole fraction as the composition variable was critical in obtaining the excellent fit of the free-solvent model. A combination of the free-solvent correction for the concentration variable coupled with models incorporating solute–solute interaction, such as a virial expansion, will be necessary to generally describe the osmotic pressure of protein solutions for all concentrations.

Key Words: **osmotic pressure, IgG, immuno-gamma globulin, hydration, globular proteins.**

INTRODUCTION

The globular proteins, such as bovine serum albumin (BSA) and immunogammaglobulins (IgG) in aqueous media have been extensively used in ultrafiltration membrane studies. However, relatively few studies have measured the osmotic pressure of these proteins at the concentrations expected at the membrane surface which can approach saturation (1–5). A number of theoretical models have been proposed for the osmotic pressure of protein solutions (1, 6–8). In particular, the virial expansion approach using mass concentration has been used extensively. The virial expansion model is insightful since the virial coefficients can be directly related to solute–solute interaction potentials for dilute solutions. But, even though protein concentrations are nearly always dilute with respect to molar concentration, the virial expansions truncated to a few terms often fail to predict osmotic pressure for the full range of concentrations (3). Furthermore, the general form of the virial expansion using standard mass or molar concentration does not account for solute–solvent binding interaction which may be a significant, and indeed, a dominant factor in many cases.

An alternate approach would be the use of the free-solvent model in cases where solvent–solute binding interaction or hydration is considered. Approximations to this model have been use for solutions with moderate protein concentrations with limited success but the insights to molecular interactions in concentrated protein solutions provided by this general model have yet to be realized. This work examines and demonstrates the effectiveness of the free-solvent model for concentrated protein solutions based on its application to concentrated IgG solution under physiological conditions in buffered solutions.

MODELING OF OSMOTIC PRESSURE FOR PROTEIN SOLUTIONS

Osmotic pressure is related to the chemical potential of species in solution. The chemical potential for species i is the partial molar Gibbs free energy. Assuming it to be a function of temperature, pressure and composition, i.e., $\mu_i = \mu_i(T, p, n_1, n_2, \cdots n_n)$, for an n component mixture (9–11), the total derivative can be written as

$$d\mu_i = \left(\frac{\partial \mu_i}{\partial T}\right)_{p,n_i} dT + \left(\frac{\partial \mu_i}{\partial p}\right)_{T,n_i} dp + \sum_{j=1}^{n} \left(\frac{\partial \mu_i}{\partial n_j}\right)_{T,p,n_{k,k\neq j}} dn_j = -\bar{S}_i\, dT + \bar{V}_i\, dp + d_{T,p}\mu_1. \quad [1]$$

[1] To whom correspondence should be addressed at 125 CB, The University of Iowa, Iowa City, IA 52242-1219. E-mail: vrodgers@icaen.uiowa.edu.

0021-9797/98 $25.00

Lewis showed that the integrated form of chemical potential for species i due to an isothermal change for any component in any system (solid, liquid, gas, ideal, nonideal) may be written in terms of fugacity as (9)

$$\mu_i(T, p, (x)) - \mu_i^0(T, p^0, (x^0)) = RT \ln \frac{f_i[T, p, (x)]}{f\,{}_i^0[T, p^0, (x^0)]}, \quad [2]$$

where f_i is the fugacity. One should note that while either $\mu_i^0(T, p^0, (x^0))$ or $f\,{}_i^0[T, p^0, (x^0)]$ are arbitrary, only one may be chosen independently. Lewis further defined relative activity as

$$a_i(T, p, (x)) \equiv \frac{f_i[T, p, (x)]}{f\,{}_i^0[T, p^0, (x^0)]} \quad [3]$$

so that

$$\mu_i(T, p, (x)) - \mu_i^0(T, p^0, (x^0)) = RT \ln a_i(T, p, (x)). \quad [4]$$

Thus relative activity has been constructed to account for both pressure changes and changes in composition in the chemical potential. Often the relative activity is expressed in terms of a composition variable, θ_i (i.e., x_i, ω_i, c_i, p_i, ρ_i, ϕ_i, etc.), and the corresponding activity coefficient, such as,

$$a_i^\theta(T, P, (\theta)) \equiv \gamma_i^\theta(T, P, (\theta))\theta_i, \quad [5]$$

where the activity coefficient can have dimensions. This equation defines the activity coefficient, γ_i^θ, which now accounts for the solution nonideality.

Many models for predicting osmotic pressure with respect to protein composition have been developed (1–8). With reference to a two compartment chamber separated by a semi-impermeable membrane, the side of the chamber containing the proteins is designated as compartment II (inside compartment) and the compartment containing only the solvent and diffusible ions, as I (outside compartment). Then, for a three-component system, setting $d\mu_i = 0$ for solvent species and denoting the solvent, solute, and ions by the subscripts 1, 2, and 3, respectively, the relative activity of the solvent, a_1, is related to the pressure difference between the two chambers at constant temperature by

$$\pi = \frac{\mu_1^{\mathrm{I}(T,P)} - \mu_1^{\mathrm{II}(T,P)}}{\bar{V}_1} = -\frac{RT}{\bar{V}_1} \ln \frac{a_1^{\mathrm{II}}}{a_1^{\mathrm{I}}} = -\frac{RT}{\bar{V}_1} \ln \frac{\gamma_1^{\mathrm{II}} x_1^{\mathrm{II}}}{\gamma_1^{\mathrm{I}} x_1^{\mathrm{I}}}. \quad [6]$$

The superscripts denote the two different chambers of the osmometer. It has been assumed in the above that the partial molar volume of the solvent is independent of pressure and composition, but Eq. [6] is otherwise remarkably free of assumptions.

A number of additional assumptions are, however, frequently made when relating Eq. [6] to experimental data. As noted above, assuming ideal solution, the activity coefficient of the solvent becomes unity and the osmotic pressure can be then approximated by the van Laar equation (6)

$$\pi = -\frac{RT}{\bar{V}_1} \ln \frac{x_1^{\mathrm{II}}}{x_1^{\mathrm{I}}}. \quad [7]$$

For a binary ideal solution in which protein is limited to only chamber II, Eq. [6] can be written in terms of the concentration of the protein as

$$\pi = -\frac{RT}{\bar{V}_1} \ln(1 - x_2^{\mathrm{II}}). \quad [8]$$

When the protein solution is dilute and ideal, the logarithmic term of Eq. [6] can be expanded into a power series and truncated to the first term to give

$$\pi \approx \frac{RT}{\bar{V}_1} x_2^{\mathrm{II}}. \quad [9]$$

The mole fraction is often inconvenient for use with real solutions. However, for dilute solutions, the mole fraction per specific volume may be approximated by mass concentration to yield the familiar van't Hoff equation

$$\pi \approx RT \frac{w_2^{\mathrm{II}}}{M_2}, \quad [10]$$

where w_2 is in grams of protein per liter of solution and M_2 is the solute molecular weight.

Virial Equation

Most often, deviation from ideality can be related to the attractive interactions of solutes and the existence of excluded volume. Using statistical mechanics, it has been shown that osmotic pressure can be expressed as an integral series expansion of concentration for dilute solutions (12). This approach has its origins from an analogy of dilute liquid theory with imperfect gas theory. While the virial expansion for the activity is generally accepted to be rigorous, the expansion for many concentration variables, such as number density, ρ, is valid only in the limit as $\rho \to 0$. Since the relationship of the virial expansion to the selected concentra-

tion variable is important, it is instructive to summarize its development. Details can be found elsewhere (12, 13).

The notation of Hill will be used throughout (12, 13). For a two-component system in which the solvent is defined as species 1 and the solute is defined as species 2, the osmotic pressure can be found via

$$e^{\pi V/kT} = \sum_{N\geq 0} \frac{Z_N(\mu_1, V, T)}{N!} z_2^N, \qquad [11]$$

where Z_N is the configurational integral which is related to the energy levels of the solute and solvent molecules and whose form is dependent on the limits of the solute activity, z_2. Here N is the number of solute molecules (species 2) in the volume, V. Defining Z_N appropriately so that $z_2 \to \rho_2$ as the solute number density, $\rho_2, \to 0$, we can take the logarithm of Eq. [11] and expand to obtain

$$\frac{\pi}{kT} = \sum_{j\geq 1} b_j(\mu_1, T) z_2^j \qquad [12]$$

where the b_j's are the coefficients of the power series in activity, z_2, and are related to the Z_N's by the semi-invariants of Thiele

$$j!Vb_j = j! \sum_n (-1)^{\sum n_i - 1} (\sum_i n_i - 1)! \prod_i \left[\frac{(Z_i/i!)^{n_i}}{n_i!} \right]. \qquad [13]$$

Equations [11]–[13] are generally accepted as rigorous for $z_2 \to \rho_2$ as $\rho_2 \to 0$. The above is completely analogous to imperfect gas theory for a single component gas in a vacuum. For this case, however, the definition of the activity has been constructed to include the contact of the solute with the solvent.

In application, the activity must be expressed in terms of a concentration variable, such as ρ_2 and an activity coefficient. Recognizing that the activity coefficient is a function of the concentration variable, the activity can be written as a power series in the variable of choice; i.e.,

$$z_2 = a_1\rho_2 + a_2\rho_2^2 + a_3\rho_2^3 + \cdots . \qquad [14]$$

Using Eq. [12] and the definition of ρ_2, it can be shown that

$$\rho_2 = \frac{N_2}{V} = z_2 \left(\frac{\partial \pi/kT}{\partial z_2} \right)_{\mu_1, T} = \sum_{j\geq 1} j b_j(\mu_1, T) z_2^j. \qquad [15]$$

Substituting Eq. [14] into Eq. [15] and requiring higher powers of ρ_2 to vanish, a relationship between the a_i's and b_i's is found, i.e.,

$$\begin{aligned} a_1 &= \quad 1 \\ a_2 &= \quad -2b_2 \\ a_3 &= -3b_3 + 8b_2^2. \\ &\vdots \\ a_n &= \quad \cdots \end{aligned} \qquad [16]$$

The osmotic pressure for this binary system can then be written in terms of a virial expansion as

$$\frac{\pi}{kT} = \rho_2 + \sum_{n\geq 2} B_n'(\mu_1, T) \rho_2^n, \qquad [17]$$

where the B_n''s are the virial coefficients which are related to b_n's by

$$\begin{aligned} B_2' &= \quad -b_2 \\ B_3' &= -4b_2^2 - 2b_3 \\ &\vdots \\ B_n' &= \quad \cdots \end{aligned} \qquad [18]$$

Clearly the virial coefficients are a function of the configurational integrals which are defined by the choice in the composition variable.

One advantage of Eq. [17] is that the virial coefficients can be related to the interaction contributions of the potential of mean force in a way similar to imperfect gases via McMillan–Mayer dilute solution theory (14). As an example, assuming spherical molecules, the second virial coefficient (in terms of molar concentration) can be expressed as (15)

$$B_2 = -\frac{N_{Av}}{2} \int_0^\infty [e^{-w_{22}/kT} - 1] 4\pi r^2 \, dr, \qquad [19]$$

where the potential energy of interaction, w_{22}, is a sum of the average short-range and screened long-range potentials acting on the solutes. The primary constraint for the use of dilute solution theory is that only short-range or valence forces are allowed between interacting solute molecules in solution. Long-range forces include electrostatic contributions, induction, dispersion, and hydrophobic forces. In general, the energy associated with long-range interaction is usually proportional to r^{-n}, where r is the surface-to-surface distance between the solutes and $n \leq 3$ (16, 17). Thus, their presence in w_{22}, of Eq. [19] will cause the second virial coefficient to diverge. However, in systems which contain both macro-ions and moderately high concentrations of micro-ions, such as proteins in saline solutions, a significant amount of Debye screening due to the ion atmosphere can result, causing the long-range potential (except hydropho-

bic) to be further reduced by a factor of $\exp(-\kappa r)$ (18, 19). In these cases, the virial expansion remains a reasonable approximation provided that the protein concentration remains moderate.

For a dilute nonideal binary solution containing a single solute and solvent with diffusible salts on the other side of the membrane, the osmotic pressure, Eq. [9], can be approximated by the virial equation (12)

$$\pi_p = RT[B_1 w_2 + B_2 w_2^2 + B_3 w_2^3 + \cdots], \qquad [20]$$

where the first virial coefficient, B_1, is $1/M_2$, B_2 ($= B_2'/M_2^2$) is the second virial coefficient, and B_3 is the third virial coefficient. The virial coefficients B_2 and B_3 have been used to represent *solute–solute* interactions, specifically the two- and three-body interactions, respectively, between protein molecules in dilute solutions. The electrolyte (species 3) contribution to osmotic pressure is usually approximated by (2)

$$\pi_D = RT\left[2\sqrt{\left(\frac{\bar{Z} w_2}{2M_2}\right)^2 + m_3^2} - 2m_3\right], \qquad [21]$$

where $\bar{Z}$ is the protein charge, and m_3 is the molal concentration of the salt. This osmotic pressure difference results from salt ion redistribution in solution and is known as the Donnan effect. The total osmotic pressure is then the sum of both contributions, i.e.,

$$\pi \approx \pi_P + \pi_D. \qquad [22]$$

At higher salt concentrations, greater than 0.1 M, however, the Donnan effect is known to be negligible (2). Models incorporating the second and higher virial coefficients have been used extensively to describe dilute protein behavior (2, 4, 20).

It has been long recognized that solute has the potential to bind solvent species in biophysical systems. Hill addresses this by treating the solute as a composite consisting of bound solvent species (subspecies) and provided an expression similar to Eq. [11] for species-bound solute in solution using a statistical mechanical approach (12). In this case, the activity is constructed so that $z \to \rho$ ($= M/V$), where M is now the total number of solute molecules of all subspecies.

Application to concentrated solutions. Despite its origin in dilute solution theory, the virial equation (Eq. [17]) is also frequently used for concentrated solutions. One approach that has been effective is to correct the number density, ρ, so that nonidealities due to the volume of the solute are considered. An example of this is the use of Scaled Particle Theory (SPT) (21, 22) which has been used with some success for high concentrated protein solutions for determining sedimentation coefficients (23).

An alternate approach is to reconsider the choice of the concentration variable to describe activity. To begin, the fugacity of a solute in solution can be described as $f_2 = z_2 kT$. From the definition of Henry's law constant, it is recognized that $f_2 \to k_2 x_2$ as $x_2 \to 0$. Here, k_2 is the Henry's law constant and x_2 is the mole fraction of species 2. Lewis and Randall argue that, from this relationship, mole fraction is the preferred concentration variable for properly describing solute concentrations (24). It is important to note that, in fact, Hill (12) begins the development of the virial equation by relating the fugacity to mole fraction and setting the limit that $x_2 \to \rho_2 \upsilon_1$ as $x_2 \to 0$, where υ_1 is the volume per molecule of pure solvent. Hill (12) then uses this relationship to develop an expression for the activity coefficient and define the configurational integral, Z_N, in Eq. [11]. While this approximation for mole fraction is certainly valid for dilute solutions, it is not for high concentrated solutes. It is, however, straightforward to derive a virial expansion for osmotic pressure in terms of mole fraction.

The significance of mole fraction as the composition variable is further justified when one recognizes that Eq. [4] is analogous to a Maxwell–Boltzmann distribution for the ideal gas case (25), where the activity coefficient is unity and the concentration variable is mole fraction, x_i. Then the mole fraction represents the fraction of species i in the system with free energy μ_i^0 (no attractive interaction).

The impact of the choice of the concentration variable at even moderate concentrations can be seen when we examine Eq. [14]. This can be demonstrated by the following example. Assume that an ideal case in which activity can be described directly as mole fraction,

$$z_2 = x_2 = \frac{N_2}{N_1 + N_2} = \frac{\rho_2}{\rho_1 + \rho_2}. \qquad [23]$$

Then, using a Taylor's series approximation Eq. [23] can be expressed as

$$z_2 = \frac{1}{\rho_1}\rho_2 - \frac{1}{\rho_1^2}\rho_2^2 + \frac{1}{\rho_1^3}\rho_2^3 + \cdots. \qquad [24]$$

Comparing the above to Eq. [14] one can see at once that the leading term in Eq. [14] is only valid as $\rho_2 \to 0$ and, thus, the approximation is not valid for higher concentrations. It should be noted that mole fraction of protein solutions is typically $O(10^{-4})$ and, thus, the above expansion is generally excellent for when no solute–solvent interaction is present. However, when the solute–solvent binding is with water (i.e., hydration), and the composite solute mole fraction is invoked, the solvent number density is reduced by a factor on the order of the hydration ratio. This can substantially increase the composite solute mole fraction. This is discussed further below.

Protein Hydration

As mentioned above, solute–solvent binding interactions have been considered in analyzing the osmotic pressure of biophysical systems. While many ions in a physiological medium bind with proteins, water is, nevertheless, substantial. This phenomenon is known as protein hydration.

There exists an extensive amount of literature on protein hydration and excellent reviews on the subject can be found [i.e. 26, 27]. The literature contains a considerable discussion on the amount of water bound and the binding forces in the hydration zone of proteins. It is generally agreed that the hydration shell, or hydration zone, is usually one or more layers of water around a protein (28). However, these layers of water are not rigidly bound to the protein. In the first monolayer, the hydrated water generally has residence times less than 10^{-9} s (with only a small fraction with rotational correlation times greater than 10^{-8} s). This is only an order-of-magnitude less than that for bulk water, suggesting that these macromolecules are only slightly constrained with respect to the remaining water which acts as solvent (29). Furthermore, in this first layer, the water has free energy of binding only on the order of kT, indicating again that the water is loosely bound. For small globular proteins in aqueous media, this first hydration layer consists of approximately 0.3 to 0.4 g H_2O/g protein. This number depends on the protein size and shape and may be slightly higher for solutions containing salts. The next few layers in the hydration zone result from cooperative interaction between the solvent molecules and the first hydration layer. Based on water–^{17}O magnetic resonance (30), the hydration zone for globular proteins, including IgG, is only about two layers of water which is equivalent to about 1 g H_2O/g protein [moles H_2O/mole protein = $O(10^4)$] for globular proteins (27, 31).

Correction for Hydration in the Concentration Variable

Free-solvent model. Using mole fraction and considering the hydrated protein as a single composite component, one may account for many of the nonidealities observed in protein solution osmotic pressure at high concentrations. Then only the free solvent is considered in the chemical potential equilibrium. The application of this concept is nearly a century old. As an example, Frazer and Myrick (in 1916) (32), in their work with aqueous sucrose solutions, suggested that the free solvent in solution exhibits near ideal behavior, provided that hydration and association are considered in determining the effective free-solvent concentration. They argued that the solvent participating in solute hydration should be considered as a part of the solute and, thus, must not be considered in determining the overall mole fraction of the free solvent in chamber II in using the van Laar equation (Eq. [7]). Much later, other researchers arrived at similar conclusions regarding solute–solvent interaction, but based their models on the van't Hoff equation which is strictly valid for dilute solutions and used it for protein solutions up to moderate concentrations by accounting for the ''interactive solvent'' (33–37).

From the above, we propose to determine the effect of these corrections on the osmotic pressure model for IgG. We begin by constructing a model based on the van Laar model (Eq. [7]) that corrects for free-solvent concentration. Alternately, a virial equation can be constructed with this concentration variable that can then account for solute–solute interaction.

To begin, the corrected free-solvent mole fraction is the initial moles of solution species minus the net moles of solvent species that are protein bound or interacting with the protein. It is assumed that the solution is made up of n distinct species. Letting species 1 be the solvent, species 2 through p be the proteins, and species $p + 1$ through n be the remaining diffusible solvent components, then the initial total moles of the solution in compartment II, $N^{II} = \Sigma_{i=1}^{n} N_i^{II}$, where i is representative of each of the n species. The final total moles of free-solvent (diffusible salts and water) in chamber II is $N_*^{II} = N^{II} - \Sigma_{i=1_{i\neq 2,p}}^{n} \Sigma_{j=2}^{p} \nu_{ij} N_j^{II} - \Sigma_{j=2}^{p} N_j^{II}$, where N_j^{II} denotes the moles of protein j in solution and ν_{ij} is the net number of moles of solution component i that is interacting with protein j to make up the new solvent-interacting protein. It may be noted that protein–protein interaction is not considered. Then, the mole fraction of free-solvent 1 in compartment II is then

$$x_1^{II} = \frac{N_1^{II} - \Sigma_{j=2}^{p} \nu_{1j} N_j^{II}}{N_*^{II} + \Sigma_{j=2}^{p} N_j^{II}} \qquad [25]$$

while in compartment I

$$x_1^{I} = \frac{N_1^{I}}{N^{I}} . \qquad [26]$$

When solute–solute interaction is not dominant, this free-solvent mole fraction can be used in the van Laar equation (Eq. [7]) to relate osmotic pressure to solute concentration. The interaction parameters, ν_{ij}, can either be used to predict osmotic pressure, or alternatively, the osmotic pressure with respect to concentration data can be used to determine the interaction parameters, ν_{ij}. We will investigate this model with data obtained for high concentrated IgG in physiologic solution. The observed osmotic pressure data are also fitted to the virial expansion model based on mass concentration truncated at the second and third virial coefficient for the purpose of comparison.

APPLICATION OF MODELS TO IgG SOLUTIONS

The osmotic pressure of IgG has been reported for various solution properties up to concentrations of 200 g/L, but this

is well below the saturation limit of IgG (38–42). Therefore, this report provides the osmotic pressure data at high concentrations of IgG in physiological aqueous solutions (pH 7.4, 0.13 M salts).

IgG is actually a heterogeneous mixture of many immunoproteins of similar structure (43). The general shape of IgG reported is that of an elliptic cylinder with cross-sectional minor semiaxis lengths of between 9.4 × 9.4 and 10.9 × 25 Å with major axis length between 231 and 240 Å and an average molecular weight of 159,000 Da (44, 45). The overall charge of each protein member making up IgG is the net result of the various basic and acidic side chains on each macromolecule. The heterogeneity of IgG is most obvious in its isoelectric chromatograph, where it was found to have prominent values between 5.8 and 8.5 (46). Thus IgG solutions at pH 7.4 have components consisting of both positively and negatively charged species.

Experimental Method

The buffer solution was prepared by dissolving a phosphate-buffered saline (No. 1000-3, Sigma, St. Louis, MO) and 20.0 mg of sodium azide (as preservative, No. S-2002, Sigma) in 1 L of deionized water. The buffer had a total salt concentration of 0.13 M (0.00239 mol salt/mole H_2O) which consisted of 0.12 M NaCl, 0.0027 M KCl, and 0.01 M phosphate buffers. The pH of the buffer solution was adjusted to 7.4 using 0.1 M NaOH and HCl. A weighed amount of IgG powder (from bovine plasma, No. G-7516, Sigma) was dissolved in a known amount of buffer solution. The solution was then stirred and stored in the refrigerator at 4°C for 2 or 3 days until it completely dissolved. The pH of the protein solution was adjusted before each run to 7.4. The solution was vigorously mixed using a vortex mixer (Vortex Genie, Model No. G-560, Fisher Scientific, St. Louis, MO) during the pH adjustment to prevent local denaturation of the protein. The pH measurements were conducted using Ross combination electrode with epoxy body (Model 8115, Orion, Boston, MA) and pH/ion meter (Accument 950, Fisher Scientific).

An osmometer was constructed based on modifications of the design described by Vilker (47). The osmometer consists of two chambers separated by a semipermeable membrane (5000 MWCO, cellulose ester, Molecular/Por, Type C, Spectrum, Laguna Hills, CA) supported between two, highly porous, stainless-steel plates, the solution chamber and the solvent chamber. The volume of the solution chamber is approximately 2.5 mL while the volume of solvent chamber is approximately

TABLE 1
Comparisons of Measured Osmotic Pressure with Model Predictions for IgG.

IgG g/(L solution)	Measured osmotic pressure	Free-solvent model		Models with no hydration correlation		Predicted pressure using virial model Eq. [20]		
		General Eq. [27]	Binary dilute Eq. [29]	van't Hoff Eq. [10]	van Laar Eq. [7]	Second coefficient from dilute data	Second and third coefficients from dilute data	Second and third coefficients from entire data
50.0	0.16	0.13	0.12	0.12	0.12	0.08	0.10	0.08
82.6	0.21	0.22	0.21	0.20	0.20	0.13	0.18	0.12
98.8	0.23	0.28	0.26	0.25	0.25	0.16	0.23	0.15
108.7	0.25	0.31	0.29	0.27	0.27	0.18	0.26	0.17
151.5	0.36	0.48	0.45	0.39	0.40	0.26	0.44	0.27
152.6	0.33	0.48	0.46	0.40	0.40	0.26	0.44	0.28
197.9	0.55	0.71	0.67	0.54	0.54	0.35	0.71	0.47
214.4	0.59	0.81	0.76	0.59	0.59	0.39	0.84	0.57
240.7	0.89	0.98	0.93	0.68	0.68	0.45	1.08	0.78
249.7	0.87	1.05	1.00	0.71	0.71	0.47	1.17	0.86
285.8	1.28	1.37	1.30	0.83	0.84	0.55	1.59	1.25
295.9	1.53	1.47	1.40	0.87	0.87	0.58	1.73	1.39
351.3	2.43	2.25	2.13	1.09	1.09	0.72	2.64	2.32
357.4	2.62	2.36	2.24	1.12	1.12	0.74	2.76	2.45
377.2	2.92	2.78	2.64	1.20	1.20	0.79	3.16	2.89
424.3	4.18	4.33	4.11	1.42	1.42	0.93	4.31	4.17
507.4		16.15	15.33			1.18	6.99	7.33
546.7		∞	∞			1.30	8.44	9.09
RMS[a]		0.035	0.041	0.245	0.244	0.303	0.045	0.022
Maximum error		0.26	0.38	2.76	2.76	3.25	0.31	0.17

Note. All Pressures in psi.
[a] Root mean square of the error between the measured pressure and that predicted.

TABLE 2
Best Fit Values for Virial Coefficients for Eq. [20]

Truncation	Virial coefficient	Value	SE	90% confidence limits	
		Based on all data			
	B_1 [mol/g]	5.74×10^{-6}	4.82×10^{-7}	4.88×10^{-6}	6.6×10^{-6}
	B_2 [L-mol/g^2]	-3.57×10^{-8}	4.71×10^{-9}	-4.41×10^{-8}	-2.78×10^{-8}
	B_3 [(L^2-mol/g^3)]	2.04×10^{-10}	9.87×10^{-12}	1.86×10^{-10}	2.21×10^{-10}
		Based on dilute data only			
After B_1	B_1 [mol/g]	6.01×10^{-6}	1.09×10^{-6}	3.97×10^{-6}	8.04×10^{-6}
After B_2	B_1 [mol/g]	3.97×10^{-6}	3.71×10^{-7}	3.19×10^{-6}	4.77×10^{-6}
	B_2 [L-mol/g^2]	4.94×10^{-9}	3.27×10^{-9}	-2.02×10^{-7}	1.19×10^{-8}
After B_3	B_1 [mol/g]	5.27×10^{-6}	9.13×10^{-7}	3.13×10^{-6}	7.40×10^{-6}
	B_2 [L-mol/g^2]	-2.29×10^{-9}	1.86×10^{-8}	-6.66×10^{-8}	2.07×10^{-8}
	B_3 [(L^2-mol/g^3)]	1.32×10^{-10}	8.68×10^{-11}	-7.2×10^{-11}	3.36×10^{-10}

25 mL. Two different pressure transducers (PX102, ranges 0–6 and 0–25 psi, Omega, Stamford, CN) were used with a digital readout. After calibration, these transducers gave an accuracy of measurement of ±0.06 and ±0.25 psi, respectively. The pressure readings for protein solutions were found to stabilize in 5–6 h. After stabilization, the pH and ionic strength of both solutions were checked.

Osmotic Pressure Data

Table 1 summarizes the data obtained for osmotic pressure as a function of IgG concentration at pH 7.4 and 0.13 M salts. The highest concentration of IgG used in this study was 424.3 g/L. A solution was also prepared for a concentration of 507.4 g/L. However, the solution immediately became glassy upon exposure to air and could not be transferred into the osmometer.

Comparisons of Models to Experimental Data

Fit of the virial expansion model with mass concentration. The osmotic pressure data from the high concentration IgG solution were used to compare the effectiveness of the models. Table 2 shows the results of the best fit values for the virial coefficients from regression (TableCurve 2D, Jandel Scientific, San Rafael, CA) of the data representing the entire concentration range as well as with data only from the dilute range. The ''virial coefficients'' obtained from the complete data can be considered to be only empirical parameters due to the limited range of validity of the virial model. From the *dilute* data fit, however, the virial coefficients obtained may be construed to be mechanistically meaningful. Thus, the virial equation (Eq. [20]) truncated to the first term, B_1, corresponds to a molecular weight of 166,190 ± 30,177 Da which is in reasonable agreement with reported values of 159,000 Da. The second virial coefficient, B_2, determined from the virial equation truncated to the second term, was compared to values determined from data obtained by other investigators using the same regression procedure (38–42). The values from this study are generally in good agreement with the values derived from data reported in the literature (Table 3). Despite the relatively large range in magnitude, all B_2 values are positive implying that the solute–solute interaction of IgG is generally repulsive.

Figure 1 shows the predictions from the various forms of the virial equation derived from dilute data for osmotic pressure over the entire range of concentration. Clearly both models, based on estimation of virial coefficients from dilute data, fail to accurately predict the osmotic pressure over the entire range of concentrations, although the expansion up to the third virial coefficient only slightly over predicts osmotic pressure at moderate concentrations. This implies that the osmotic pressure of IgG, based on virial expansion with a composition variable in units of grams per liter of solution, has concentration-dependent virial coefficients. It has been shown that, for high concentrations, as many as seven virial coefficients are necessary to describe the observed osmotic pressure (3). Despite the fact that the virial expansion regressed over the *entire* concentration range fits very well, it must be recognized this represents only an empirical fit of the polynomial in Eq. [20].

Free-solvent model. We consider IgG to be a single protein with hydration that can be characterized by the single stoichiometric parameter, ν_{12}. Also, since the salt concentration is moderate, the Donnan effect is negligible and we assume, as a first approximation, that the counterion molar ratio to protein in the hydration zone can be represented by a single parameter, ν_{32}. Substituting these values into Eq. [25] and using the van Laar equation (Eq. [7]), we obtain

$$\pi \approx \frac{RT}{\bar{V}_1} \ln\left\{\frac{(N_1^{II} + (1 - \nu_{12} - \nu_{32})N_2^{II} + N_3^{II})N_1^{I}}{(N_1^{II} - \nu_{12}N_2^{II})N^{I}}\right\}. \quad [27]$$

TABLE 3
Comparison of B_2 with Values Determined from the Literature

	IgG source	pH	Salt concn	B_2	Error	90% confidence limit	
				[L-mol/g^2 × 10^9]			
This work	Bovine	7.4	0.13 M	4.93	3.27	−2.02	11.92
Thomas and Brown [42]	Bovine	—	0.19%	8.09	3.05	2.42	13.81
Schultz *et al.* [41]	Bovine	7.0	0.15 M	37.54	1.44	33.4	41.68
Ingerslev *et al.* [40]	Human	—	—	4.57	11.20	−18.05	27.2
Scatchard *et al.* [39]	Human	—	0.15 M	10.15	1.49	6.9	13.36
Oncley *et al.* [38]	Human			8.93	2.72	1.08	16.78

Equation [27] was best fit to the measured osmotic pressure data by using nonlinear regression (TableCurve 2D) on the parameters ν_{12} and ν_{32}. Figure 2 shows the fit of the free-solvent model for the IgG solution when hydration is taken into consideration. The ideal and ideal dilute osmotic pressure predictions are also shown. As can be seen, the free-solvent model provides a remarkable fit for all concentrations while the ideal and ideal dilute models are accurate only to 240 g/L. The resulting value for ν_{12} is 1.11 ± 0.041 g H_2O/g IgG (9803 ± 366 mol H_2O/mole IgG) with a 95% confidence range between 1.01 and 1.20 g H_2O/g IgG. The parameter ν_{32} has a value of 24.31 ± 0.97 mol salts/mole IgG with a 95% confidence range between 22.24 and 26.39 mol salts/mole IgG. The narrow 95% confidence range of the coefficients and excellent fit of the osmotic pressure data imply that the parameters ν_{12} and ν_{32} have physical significance. Indeed, the value of 1.11 g H_2O/g IgG for the parameter ν_{12} is consistent with NMR studies that imply that about 1 g H_2O/g protein, or two layers of water, reside in the hydration zone (27).

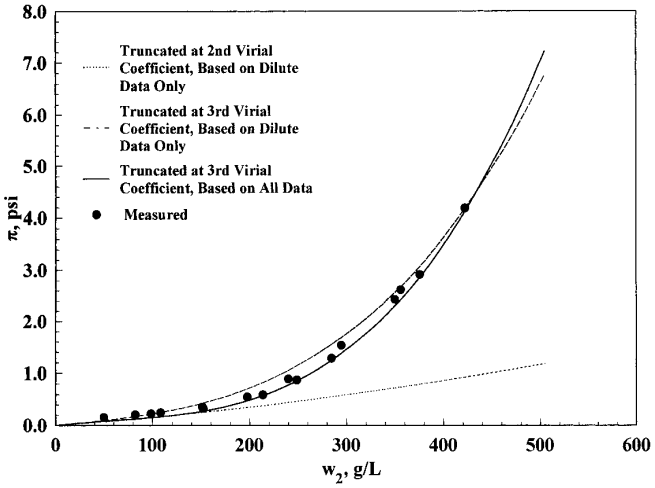

FIG. 1. Osmotic pressure versus IgG concentration at 25°C, pH 7.4, and 0.13 M salt. The dashed curves are based on virial coefficients derived from dilute solution data. The solid curve is a third order polynomial fit only.

Taking the ratio of the thus determined ν_{12} and ν_{32} results in a value of 0.00239 mol salt/moles H_2O in the hydration zone. This happens to be the same mole ratio as in the free-solvent under these solution conditions. Thus ν_{32} can be written as a constant, α, times ν_{12} and Eq. [27] can be represented by a single adjustable parameter, ν_{12}. Then

$$\pi \approx \frac{RT}{\bar{V}_1} \ln\left\{\frac{(N_1^{II} + (1 - \nu_{12}(1+\alpha))N_2^{II} + N_3^{II})N_1^{I}}{(N_1^{II} - \nu_{12}N_2^{II})N^{I}}\right\}. \quad [28]$$

The free-solvent model can also be written in terms of the solute mole fraction. Since the salt to water mole ratio is the same in both the solvent and in the hydrated layers, the free-solvent model for a dilute binary solution approximation may be used (Eq. [9]). Then,

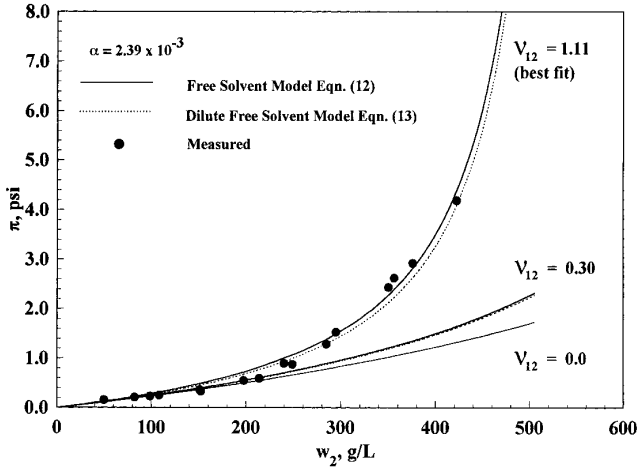

FIG. 2. Osmotic pressure versus IgG concentration at 25°C, pH 7.4, and 0.13 M salt. Curves represent the free-solvent model and dilute free-solvent model for both uncorrected and hydration-corrected mole fractions. α is the molar ratio of salt to water in both the hydration zone and in the solvent. The parameter ν_{12} is the mass ratio of water in the hydration zone to IgG. The values of ν_{12} equal to 0, 0.3 and 1.11 represent the ideal case with no hydration, free-solvent model with one monolayer of water, and our regressed value of hydration (approximately two layers of water) for the free-solvent model, respectively.

$$\pi \approx \frac{RT}{\bar{V}_1} \frac{N_2^{II}}{N_*^{II} + N_2^{II}}. \quad [29]$$

Figure 2 shows that the result of using Eq. [29] with the previously regressed value for ν_{12} is indistinguishable from the result of using Eq. [28]. One major conclusion that can be gleaned from this analysis is that the osmotic pressure behaves ideally with respect to the *free-solvent* mole fraction in solution. Thus the *solute–solvent* binding interaction may account for the dominant solution nonideality observed in this system.

Table 1 summarizes the result from the various models investigated in this study. As can be seen, the free-solvent models that correct for hydration provide osmotic pressure values in excellent agreement throughout the entire concentration range. The average error in prediction is approximately ± 0.035 and ± 0.041 psi for the general free-solvent model and the free-solvent model with the binary dilute solution assumption, respectively, for the *single* constant value of ν_{12}. This is below our error in measurement of ± 0.06 psi.

Before it can be assumed that this single parameter, ν_{12}, is a constant throughout the composition range, it is appropriate to determine the sensitivity of osmotic pressure with respect to ν_{12}. Figure 2 shows that the result of forcing ν_{12} to the value representative of a single hydration layer ($\sim$0.3 g H_2O/g protein) provides an excellent fit for data up to about 214 g/L. Indeed, the intermediate osmotic pressure values are better fit by this value. Using Eq. [29] as the representation of the osmotic pressure model

$$\frac{\partial \pi}{\partial \nu_{12}} = \frac{RT}{\bar{V}} \left(\frac{x_2^{II}}{x_1^{II}} - (1 + \alpha) x_2^{II} \right) = \frac{RT}{\bar{V}} \frac{(x_2^{II})^2}{x_1^{II}} \approx \frac{RT}{\bar{V}} (x_2^{II})^2. \quad [30]$$

Clearly, for very dilute solutions, $x_1^{II} \to 1/(1 + \alpha)$ and the osmotic pressure is independent of the value of ν_{12}. However, the osmotic pressure has a dependency for high concentrations that is two orders-of-magnitude higher than that for dilute concentrations. At 214 g/L, varying ν_{12} from zero to the value representative of 0.3 g H_2O/g protein (one monolayer of H_2O) increases the pressure by less than the error in the measurement. On the other hand, changing ν_{12} from the value representing 0.3 g H_2O/g IgG to that representing 1.11 g H_2O/g IgG (approximately two layers of H_2O) causes a 0.14 psi deviation. This difference might be what is shown in Fig. 2 at the concentrations around 200 g/L, implying that the actual number of hydration shells may be concentration dependent. Hill (12) discusses a similar concentration dependency on the binding equilibrium in his model. Further, the insensitivity of the osmotic pressure to ν_{12} at low concentrations would result in considerable error in a value for ν_{12} determined from data in the dilute region. However, the high sensitivity of osmotic pressure on concentrations over 250 g/L and the excellent fit for this data imply that, at least in the high concentration region, ν_{12} is constant and can be determined accurately.

Other researchers (35–37) used linear extrapolation of inverse pressure with respect to g H_2O/g protein and estimated values of 1.57 g H_2O/g hemoglobin and values greater than 3 g H_2O/g BSA from reported osmotic pressure data for these two proteins. Using our data we found that this intercept method can vary by as much as a factor of 2, depending on the highest concentration data used and the extrapolating function.

The value of ν_{12} determined above implies that the limit of solubility for IgG under these conditions corresponds to about 546.7 g/L. Returning to the experimental data, it may be recalled that a sample representing 507.4 g/L could not be used because it became glassy upon exposure to air. Based on the amount of water used to produce this sample, the concentration of the sample could have reached this estimated saturation value with a loss of only 0.29 g H_2O. The free-solvent model (Eq. [28]) estimates the osmotic pressure of this concentration to be 16 psi.

It should be noted that the models above differ from that used by Fullerton *et al.* (36) in that mole fraction is used here as a measure of composition and not molal concentration. We have found that the composition variable in terms of molal concentration results in a lack of fit for our highest concentration data, similar to that observed by Fullerton *et al.* (36). This is because the difference in the two concentration variables is significant at concentrations near saturation.

CONCLUSION

Measurements to near saturation of IgG in phosphate-buffered solution at pH 7.4 and total salt concentration of 0.13 M were determined. The resulting data were used to compare various models relating osmotic pressure to concentration. The free-solvent model, which assumes nonidealities observed in osmotic pressure are dominated by solute–solvent interaction, was used and compared to high concentration osmotic pressure data for IgG in physiologic solution. The results demonstrated that the free-solvent model fits excellently. Further, the amount of hydration determined from this free-solvent method corresponded to that observed for other globular proteins at high concentrations using water–^{17}O magnetic resonance. This result gives some validity to the regressed hydration parameter. These results imply that the hydrated composite macromolecules have negligible attractive force between each other even when at high concentrations. However, a more complete model of osmotic pressure of high concentrated protein solutions may require consideration of both solute–solute and solute–solvent in-

teraction. This may be accomplished by using the modified concentration variable in a virial expansion.

APPENDIX: NOMENCLATURE

a_i Relative activity of species i
a_i ith coefficient of power series in Eq. [14]
B_1 Inverse molecular weight of solute in Eq. [20]
B_2 Second virial coefficient in Eq. [20]
B_3 Third virial coefficient in Eq. [20]
B'_n nth virial coefficient in Eq. [17]
b_j jth coefficient of the power series in activity, z_2
f_i Fugacity of species i
k Boltzmann constant
kT Thermal energy
M Total number of solute molecules of all subspecies
M_i Molecular weight of species i
m_i Molal concentration
N Number of solute molecules (species 2)
N_{Av} Avogadro's number
N^{II}_* Final available total solvent moles
N^j Initial total moles of solvent in compartment j
N^j_i Initial total moles of solvent species i in compartment j
n Number of solvent species
p Number of protein species
R Ideal gas constant
r Surface-to-surface distance between the solutes
T Temperature
V Volume
$\bar{V}_i$ Molar volume
w_2 Grams of solute 2 per liter of solution
w_{22} Potential energy of interaction
x_i Mole fraction
$\bar{Z}$ Protein charge
Z_N Configurational integral
z Activity
z_2 Solute activity

Greek

α Moles salt/moles water
γ_i Activity coefficient
μ_i Chemical potential of species i
μ^0_i Standard state free energy
π Osmotic pressure
ρ Number density
θ Composition variable
υ_1 Volume per molecule of pure solvent
ν_{ij} Net number of moles of solvent component i that is interacting with protein j

Superscripts

I Compartment I
II Compartment II
o Standard state

Subscripts

p Protein contribution
D Donnan contribution
1 Solvent
2 Protein
3 Ions

ACKNOWLEDGMENT

Financial support was provided by the USDA.

REFERENCES

1. Scatchard, G., *J. Am. Chem. Soc.* **68,** 2315 (1946).
2. Vilker, V. L., Colton, C. K., and Smith, K. A., *J. Colloid Interface Sci.* **79,** 548 (1981).
3. Ross, P. D., and Minton, A. P., *J. Mol. Biol.* **112,** 437 (1977).
4. Haynes, C. A., Tamura, K., Körfer, H. R., Blanch, H. W., and Prausnitz, J. M., *J. Phys. Chem.* **96,** 902 (1992).
5. Pjura, P. E., Paulaitis, M. E., and Lenhoff, A. M., *AIChE J.* **41,** 1005 (1995).
6. Tanford, C., ''Physical Chemistry of Macromolecules.'' Wiley, New York, 1961.
7. Kirkwood, J. G., ''Proteins,'' Gordon and Breach, New York, 1967.
8. Casassa, E. F., and Eisenberg, H., *in* ''Advances in Protein Chemistry,'' Vol. 19, pp. 287–395. Academic Press, New York, 1964.
9. Prausnitz, J. M., Lichtenthaler, R. N., and de Azevedo, E. G., ''Molecular Thermodynamics of Fluid-Phase Equilibria,'' 2nd ed. Prentice-Hall, Englewood Cliffs, NJ, 1986.
10. Denbigh, K., ''The Principles of Chemical Equilibrium.'' 4th ed. Cambridge Univ. Press, Cambridge, 1981.
11. Kirkwood, J. G., and Oppenheim, I., ''Chemical Thermodynamics.'' McGraw–Hill, New York, 1961.
12. Hill, T. L., ''An Introduction to Statistical Thermodynamics.'' Dover, New York, 1986.
13. Hill, T. L., ''Statistical Mechanics.'' Dover, New York, 1956.
14. McMillan, W. G., and Mayer, J. E., *J. Chem. Phys.* **13,** 276 (1945).
15. Stigter, D., and Hill, T. L., *J. Phys. Chem.* **3,** 551 (1959).
16. Adamson, A. W., ''A Textbook of Physical Chemistry.'' Academic Press, New York, 1973.
17. Israelachvili, J., ''Intermolecular and Surface Forces.'' 2nd ed. Academic Press, London, 1992.
18. Hirschfelder, J. O., Curtiss, C. F., and Bird, R. B., ''Molecular Theory of Gases and Liquids.'' Wiley, New York, 1954.
19. Tombs, M. P., and Peacocke, A. R., ''The Osmotic Pressure of Biological Macromolecules.'' Clarendon Press, Oxford, 1974.
20. Wang, L., and Bloomfield, V. A., *Macromolecules* **23,** 194 (1990).
21. Gibbons, R. M., *Mol. Phys.* **17,** 81 (1969).
22. Gibbons, R. M., *Mol. Phys.* **18,** 809 (1970).
23. Chatelier, R. C., and Minton, A. P., *Biopolymers* **26,** 524 (1987).
24. Lewis, G. N., and Randall, M., ''Thermodynamics,'' second ed. McGraw–Hill, New York, 1961.

25. Glasstone, S., ''Textbook of Physical Chemistry,'' 2nd ed. Van Nostrand, Toronto, 1946.
26. Kuntz, I. D., Jr., and Kauzmann, W., *Adv. Protein Chem.* **28,** 239 (1974).
27. Rupley, J. A., and Careri, G., *Adv. Protein Chem.* **41,** 38 (1991).
28. Israelachvili, J., ''Intermolecular and Surface Forces,'' 2nd ed. Academic Press, London, 1992.
29. Otting, G., Liepinsh, E., and Wüthridh, K., *Science* **254,** 974 (1991).
30. Halle, B., Andersson, T., Forsén, S., and Lindman, B., *J. Am. Chem. Soc.* **103,** 500 (1981).
31. Dedushenko, V. F., *Tr. Turkmensk. Med. Inst.* **11,** 169 (1962).
32. Frazer, J. C., and Myrick, R. T., *J. Am. Chem. Soc.* **38,** 1907 (1916).
33. Ling, G. N., Ochsenfeld, M. M., Walton, C., and Bersinger, T. J., *Phys. Chem. Phys.* **12,** 3 (1980).
34. Ling, G. N., ''In Search of the Physical Basis of Life.'' Plenum, New York, 1984.
35. Cameron, I. L., and Fullerton, G. D., *Biochem. Cell Biol.* **68,** 894 (1990).
36. Fullerton, G. D., Zimmerman, R. J., Cantu, C., and Cameron, I. L., *Biochem. Cell Biol.* **70,** 1325 (1992).
37. Kanal, K. M., Fullerton, G. D., and Cameron, I. L., *Biophys. J.* **66,** 153 (1994).
38. Oncley, J. L., Scatchard, G., and Brown, A., *J. Phys. Chem.* **51,** 184 (1947).
39. Scatchard, G., Gee, A., and Weeks, J., *J. Phys. Chem.* **58,** 783 (1954).
40. Ingerslev, P., Larsen, O. A., and Lassen, A., *Scand. J. Clin. Lab. Invest.* **18,** 431 (1966).
41. Schultz, J. S., Valentine, R., and Choi, C. Y., *J. Gen. Physiol.* **73,** 49 (1979).
42. Thomas, L. A., and Brown, S. A., *Am. J. Vet. Res.* **53,** 2241 (1992).
43. Stevenson, G. T., *in* ''Structure and Function of Plasma Proteins'' (A. Allison, Ed.), Vol. 1, p. 223 (1974).
44. Kratky, O., and Paletta, B., *Angew. Chem.* **67,** 602 (1955).
45. Kratky, O., Iz, I. P., Schmitz, P. J., and Oberdorfer, R., *Z. Naturforch.* **18B,** 180 (1963).
46. Nel, R. G., Oppenheim, S. F., and Rodgers, V. G. J., *Biotechnol. Prog.* **10,** 539 (1994).
47. Vilker, V., Ph.D. Thesis, MIT, 1976.