Determination of the Equilibrium Constants of Associating Protein Systems. II. Evaluation of Virial Coefficients Derived from the Casassa and Eisenberg Definition*

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ABSTRACT: The equilibrium constants of reversibly interacting polymer systems may be obtained using Casassa and Eisenberg's definition of the modified Steiner's (Steiner, R. F. (1952), *Arch. Biochem. Biophys. 39*, 333) equation (Jeffrey, P. D., and Coates, J. H. (1966), *Biochemistry 5*, 489). The effect of the unit charge of the monomeric species on the determination of the equilibrium constants of associating protein systems is readily computed by stepwise polynomial regression analysis (P. W. Chun, M. Fried, and K. S. Yee, submitted for publication). The present investigation examines the theoretical determination of virial coefficients for polymerizing protein systems allowing for the effect of unit charge. Also outlined is a detailed theoretical treatment of $\bar{Q}^*/M^*_1 = \Delta\bar{\alpha}^*_\omega$, a determinant of the virial coefficient which may be experimentally evaluated from $\bar{M}_w$ as a function of concentration in a nonideal system.

Many protein systems undergo reversible association reactions in aqueous solution. In a system at chemical equilibrium, monomers and higher polymers must always coexist at all points within the reaction boundaries.

$$n[\mathrm{A}] \rightleftharpoons [\mathrm{A}]_{ni}$$

or

$$\bar{M}_w = \sum_i [\mathrm{A}_i]m^2/\sum_i [\mathrm{A}_i]m_i$$[1]

Detailed theoretical treatments of these polymerization reactions have been previously developed (Steiner, 1952; Gilbert, 1955, 1963; Adams and Fujita, 1963; Gilbert and Jenkins, 1963; Adams and Williams, 1964; Adams, 1965; Adams and Filmer, 1966; Jeffrey and Coates, 1966). In an attempt to predict the theoretical behavior of interacting protein systems, numerous physicochemical studies of such polymerizations have been undertaken (Rao and Kegeles, 1958; Bethune and Kegeles, 1961; Nichol and Bethune, 1963; Nichol *et al.*, 1964; Kegeles and Sarfare, 1965; Payens and Schmidt, 1965; Sarfare *et al.*, 1966; Bethune and Grillo, 1967).

A previous paper from this laboratory (P. W. Chun, M. Fried, and K. S. Yee, submitted for publication) presented a stepwise polynomial regression analysis to determine the mode of association and the equilibrium constants of reversibly interacting protein systems, using $\bar{M}_w$ as a function of concentration. The treatment was based on ideal systems in which the virial coefficients are assumed to approach zero.

The present communication describes a theoretical treatment of the virial coefficients derived from the Casassa and Eisenberg (1960, 1961) definition in nonideal solutions. With minor modifications to accommodate each specific model system and to compensate for the effect of charge, the stepwise polynomial regression analysis can be applied to the numerical evaluation of the virial coefficients of associating protein systems.

The Theoretical Examination of Virial Coefficients Derived from the Casassa and Eisenberg Definition. The Casassa and Eisenberg (1960, 1961) definition of sedimentation equilibrium in a two-component system leads to the following expression

$$M^*_2(1 - \bar{v}^*_{2\rho})\frac{\omega^2}{2RT}\left[\frac{\mathrm{d}\ln m_2}{\mathrm{d}x^2}\right]^{-1} = 1 + m_2\left[\sum_i(\nu^{*2}_{2i}/m_i) + \beta^*_{22}\right] \quad (1)$$

where m_i and m_2 are molalities of species and components, M^*_2 is the molecular weight of component 2, and β^*_{22} is the derivative of the logarithm of the activity coefficient with respect to m_2. The molecular weight obtained in sedimentation equilibrium experiments depends on the ratio of the concentration gradient to the concentration at any point (x), and only a property proportional to the concentration C^* need be measured. $L^*_2 = (1 - \bar{v}^*_{2\rho})(\omega^2/2RT)$, and $\nu^*_{2\pm}$ is the number of moles of ionic species comprising component 2. $m_\pm$ is the activity coefficient of m_+ and m_- and asterisks (*) denote quantities consistent with the Casassa and Eisenberg definition which takes the effect of charge

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[1] $[\mathrm{A}_i]$ = molar concentration of species and degree of association i. m_i = molecular weight of species i.

into consideration. Assuming eq 1 to be true for the ith component and taking the molalities of the species to be $m_i = C^*_i/M^*_i$, the equation may be stated in the following general form

$$M^*_i L^*_i \left[\frac{\mathrm{d}\ln m^*_i}{\mathrm{d}x^2}\right]^{-1} = 1 + m^*_i\left[\frac{\nu^{*}_{i+}{}^2}{m_+} + \frac{\nu^{*}_{i-}{}^2}{m_-} + \beta^*_{ii}\right] \quad (1a)$$

or

$$M^*_i L^*_i \left[\frac{\mathrm{d}\ln C^*_i/M^*_i}{\mathrm{d}x^2}\right]^{-1} = 1 + \frac{C^*_i}{M^*_i}\left[\frac{\nu^{*}_{i+}{}^2}{m_+} + \frac{\nu^{*}_{i-}{}^2}{m_-} + \beta^*_{ii}\right] \quad (1b)$$

and since

$$\left[\frac{\mathrm{d}\ln C^*_i/M^*_i}{\mathrm{d}(x^2)}\right]^{-1} = \frac{1}{C^*}\frac{\mathrm{d}C^*_i}{\mathrm{d}(x^2)}$$

eq 1b becomes

$$M^*_i L^*_i = \left[1 + \frac{C^*_i}{M^*_i}\left(\frac{\nu^{*}_{i+}{}^2}{m_+} + \frac{\nu^{*}_{i-}{}^2}{m_-}\right) + \beta^*_{ii}\right]\frac{1}{C^*}\frac{\mathrm{d}C^*_i}{\mathrm{d}(x^2)} \quad (1c)$$

where $i = 1, 2, 3\ldots$ It is assumed that the partial specific volumes of all the constituents are the same, even though the Casassa and Eisenberg definition shows small quantitative differences.

Letting

$$Z_i = \left[\frac{\nu^{*}_{i+}{}^2}{m_+} + \frac{\nu^{*}_{i-}{}^2}{m_-}\right] = i^2\left[\frac{\nu^{*}_{1+}{}^2}{m_+} + \frac{\nu^{*}_{1-}{}^2}{m_-}\right] = i^2 Z$$

and from eq 1c and the Appendix to this paper

$$\frac{\mathrm{d}C^*}{\mathrm{d}(x^2)} = \sum_i\left[\frac{M^*_i C^*_i L^*}{\left(1 + \frac{C^*_i}{M^*_i}[Z_i + \beta^*_{ii}]\right)}\right] \quad (1d)$$

Defining terms from eq 1c

$$(\overline{M}_{\mathrm{w}})^*_{\mathrm{app}} = \frac{1}{C^*}\sum_i\left[\frac{M^*_i C^*_i}{1 + \frac{C^*_i Z_i}{M^*_i} + \frac{C^*_i\beta^*_{ii}}{M^*_i}}\right] \quad (1e)$$

giving

$$\frac{\mathrm{d}C^*}{C\mathrm{d}(x^2)} = L^*(\overline{M}_{\mathrm{w}})^*_{\mathrm{app}} \quad (1f)$$

Since

$$\frac{1}{L^*}\frac{\mathrm{d}\ln C^*}{\mathrm{d}(x^2)}$$

is a function of the distance (x) in sedimentation equilibrium, this equation satisfies sedimentation equilibrium experiments on component i in which a monomeric species of unit charge is equilibrated with the other species present. For mathematical simplicity, we assume the following terms

$$M^*_i = iM^*_i$$

$$\frac{C^*_i}{M^*_i}(Z_i) = iC^*_i\left(\frac{Z}{M^*_i}\right)$$

Thus, eq 1f takes the following general form

$$(\overline{M}_{\mathrm{w}})^*_{\mathrm{app}} = \frac{1}{C^*}\sum_i\left[\frac{M^*_i C^*_i}{\left(1 + iC^*_i\frac{Z}{M^*_i}\right) + \left(i\,\frac{C^*_i\beta^*_{ii}}{M^*_i}\right)}\right] \quad (2)$$

$$(\overline{M}_{\mathrm{w}})^*_{\mathrm{app}} = \frac{1}{C^*}\left[\frac{M^*_1 C^*_1}{\left(1 + C^*_1\frac{Z}{M^*_1}\right) + \left(\frac{C^*_1\beta^*_{11}}{M^*_1}\right)} + \frac{M^*_2 C^*_2}{\left(1 + 2C^*_2\frac{Z}{M^*_2}\right) + \left(\frac{2C^*_2\beta^*_{22}}{M^*_2}\right)} + \ldots + \frac{M^*_n C^*_n}{\left(1 + nC^*_n\frac{Z}{M^*_n}\right) + \left(\frac{nC^*_n\beta^*_{nn}}{M^*_n}\right)}\right] \quad (2a)$$

This equation describes a system in which monomeric, dimeric, trimeric, and higher polymeric species coexist in equilibrium. In order to simplify the equation, the binomial theorem and approximation is used. Letting

$$\left[\frac{1}{1 + iC^*_i\frac{Z}{M^*_i}}\right] \approx \left(1 - iC^*_i\frac{Z}{M^*_i}\right)$$

where

$$iC^*_i\frac{Z}{M^*_i} \ll 1$$

and

$$\left[\frac{1}{1 + \frac{iC^*_i\beta^*_{ii}/M^*_i}{1 + iC^*_i\frac{Z}{M^*_i}}}\right] \approx \left(1 - \frac{iC^*_i\beta^*_{ii}/M^*_i}{1 + iC^*_i\frac{Z}{M^*_i}}\right)$$

where

$$\frac{iC^*{}_i\beta^*{}_{ii}/M^*{}_i}{1 + iC^*{}_i\dfrac{Z}{M^*{}_i}} \ll 1$$

Equation 2 becomes

$$(\bar{M}_{\mathrm{w}})^*{}_{\mathrm{app}} \approx \frac{1}{C^*}\sum_i\left[M^*{}_iC^*{}_i\left(1 - iC^*{}_i\frac{Z}{M^*{}_i}\right)\right] \times \left[1 - \frac{iC^*{}_i\beta^*{}_{ii}}{M^*{}_i}\left(1 - iC^*\frac{Z}{M^*{}_i}\right)\right] \quad (3)$$

Letting

$$(\bar{M}_{\mathrm{w}}{}^0)^*{}_{\mathrm{app}} = \frac{1}{C^*}\sum_i M^*{}_iC^*{}_i\left(1 - iC^*{}_i\frac{Z}{M^*{}_i}\right) \quad (3a)$$

where $(\bar{M}_{\mathrm{w}}{}^0)^*{}_{\mathrm{app}}$ represents an apparent molecular weight of i species affected by unit charge of monomer (see Appendix for numerical solution (P. W. Chun, M. Fried, and K. S. Yee, submitted for publication)).

Equation 3 takes the following general form

$$(\bar{M}_{\mathrm{w}})^*{}_{\mathrm{app}} = (\bar{M}_{\mathrm{w}}{}^0)^*{}_{\mathrm{app}} - \frac{1}{C^*}\sum_i M^*{}_iC^*{}_ii\left(1 - iC^*{}_i\frac{Z}{M^*{}_i}\right)^2\frac{\beta^{**}{}_{ii}}{M^*{}_i}$$

Letting

$$\bar{Q}^* = \frac{1}{C^*}\sum_i C^*{}_i{}^2i\left(1 - iC^*{}_i\frac{Z}{M^*{}_i}\right)^2\beta^*{}_{ii}$$

$$\bar{Q}^* \approx \frac{1}{C^*}\sum_i C^*{}_i{}^2i\left(1 - 2iC^*{}_i\frac{Z}{M^*{}_i}\right)\beta^*{}_{ii} \text{ (see eq 2)} \quad (4)$$

$$\begin{aligned}\bar{Q}^* = \frac{1}{C^*}\Bigg[&C^*{}_1{}^2\left(1 - C^*{}_1\frac{Z}{M^*{}_1}\right)\beta^*{}_{11} + \\ &2C^*{}_2{}^2\left(1 - 4C^*{}_2\frac{Z}{M^*{}_2}\right)\beta^*{}_{22} + \\ &3C^*{}_3{}^2\left(1 - 6C^*{}_3\frac{Z}{M^*{}_3}\right)\beta^*{}_{33} + \\ &4C^*{}_4{}^2\left(1 - 8C^*{}_4\frac{Z}{M^*{}_4}\right)\beta^*{}_{44} + \\ &\ldots nC^*{}_n{}^2\left(1 - 2nC^*{}_n\frac{Z}{M^*{}_n}\right)\beta^*{}_{nn}\Bigg]\end{aligned} \quad (4a)$$

In order to make eq 4a consistent with Steiner's (1952) notation, we assume $[\mathrm{A}_i] = m_i = [C^*{}_i/M^*{}_i]$. A similar equation must be written to compensate for the effects of charge and of the virial coefficient on the evaluation of the equilibrium constants.

$$\begin{aligned}\bar{Q}^* = \frac{1}{C^*}[&C^*{}_1{}^2(1 - [\mathrm{A}_1]Z)\beta^*{}_{11} + \\ &2C^*{}_2{}^2(1 - 8[\mathrm{A}_2]Z)\beta^*{}_{22} + 3C^*{}_3(1 - 18[\mathrm{A}_3]Z)\beta^*{}_{33} + \\ &4C^*{}_4(1 - 32[\mathrm{A}_4]Z)\beta^*{}_{44} + \ldots + \\ &nC^*{}_n{}^2(1 - 2n^2[\mathrm{A}_n]Z)\beta^*{}_{nn}]\end{aligned}$$

$$\begin{aligned}\bar{Q}^* = \frac{C^*{}_1{}^2}{C^*}\Bigg[&(1 - [\mathrm{A}_1]Z)\beta^*{}_{11} + \\ &2\frac{C^*{}_2{}^2}{C^*{}_1{}^2}(1 - 8[\mathrm{A}_2]Z)\beta^*{}_{22} + 3\frac{C^*{}_3{}^2}{C^*{}_1{}^2}(1 - 18[\mathrm{A}_3]Z)\beta^*{}_{33} + \\ &4\frac{C^*{}_4{}^2}{C^*{}_1{}^2}(1 - 32[\mathrm{A}_4]Z)\beta^*{}_{44} + \\ &\ldots.\ n\frac{C^*{}_n{}^2}{C^*{}_1{}^2}(1 - 2n^2[\mathrm{A}_n]Z)\beta^*{}_{nn}\Bigg]\end{aligned} \quad (4b)$$

Recalling from the Appendix that $[\mathrm{A}_2] = k_2[\mathrm{A}_1]^2$, $[\mathrm{A}_3] = k_2k_3[\mathrm{A}_1]^3$, etc.

$$\begin{aligned}\bar{Q}^* = \frac{C^*{}_1{}^2}{C^*{}_1}\Bigg[&(1 - [\mathrm{A}_1]Z)\beta^*{}_{11} + \\ &2\frac{C^*{}_2{}^2}{C^*{}_1{}^2}(1 - 8k_2[\mathrm{A}_1]^2Z)\beta_{22} + \\ &3\frac{C^*{}_3{}^2}{C^*{}_1{}^2}(1 - 18k_2k_3[\mathrm{A}_1]^3Z)\beta^*{}_{33} + \\ &4\frac{C^*{}_4{}^2}{C^*{}_1{}^2}(1 - 32k_4k_3k_2[\mathrm{A}_1]^4Z)\beta^*{}_{44} + \ldots + \\ &n\frac{C^*{}_n{}^2}{C^*{}_1{}^2}(1 - 2n^2k_n\ldots k_2[\mathrm{A}_1]^nZ)\beta^*{}_{nn}\Bigg]\end{aligned}$$

Let $x^*{}_1 = (C^*{}_1/C^*)$ and since $[\mathrm{A}_1] = x^*{}_1C^*/M^*{}_1$

$$\left(\frac{C^*{}_i}{C^*{}_1}\right)^2 = \left[\frac{\mathrm{A}_iiM^*{}_i}{\mathrm{A}_11M^*{}_i}\right]^2 = [ik_2k_3\ldots k_i(\mathrm{A}_1)^{i-1}]^2 = \left[\prod_{j=2}^{i} k_j(\mathrm{A}_1)^{i-1}\right]^2 \quad (4c)$$

and

$$C^*{}_i{}^2 = m^2{}_i[M_i]^* = [\mathrm{A}_i]^2[M_i]^* = [\mathrm{A}_i]^2iM_1$$

Thus eq 4b becomes

$$\begin{aligned}\frac{\bar{Q}^*}{x^*{}_1{}^2} = C^*[&(1 - [\mathrm{A}_1]Z)\beta^*{}_{11} + \\ &4k_2{}^2[\mathrm{A}_1](1 - 8k_2[\mathrm{A}]^2Z)\beta^*{}_{22} + \\ &9k_2{}^2k_3{}^2[\mathrm{A}_1]^4(1 - 18k_2k_3[\mathrm{A}_1]^3Z)\beta^*{}_{33} + \\ &16k^2{}_2k^2{}_3k^2{}_4[\mathrm{A}_1]^6(1 - 32k_4k_3k_2[\mathrm{A}_1]^4Z)\beta^*{}_{44} + \ldots]\end{aligned} \quad (4d)$$

Determination of Virial Coefficients, $\bar{Q}^{*0}$, Independent of Unit Charge of Monomeric Species. It should be noted that if

$$\left[iC^*{}_i\frac{Z}{M^*{}_i} + iC^*{}_i\frac{\beta^*{}_{ii}}{M^*{}_i}\right] \ll 1$$

(see eq 2a and 2c,) then eq 2 becomes

$$(\bar{M}_w)^*_{\mathrm{app}} = \frac{1}{C^*}\sum_i M^*_i C^*_i\left[1 - iC^*_i\frac{Z}{M^*_i} + iC^*_i\frac{\beta^*_{ii}}{M^*_i}\right]$$

$$(\bar{M}_w)^*_{\mathrm{app}} = (\bar{M}_w{}^0)^*_{\mathrm{app}} - \frac{1}{C^*}\sum_i iC^*_i{}^2\beta^*_{ii}$$

Letting

$$\bar{Q}^{*0} = \frac{1}{C^*}\sum_i [iC^*_i{}^2\beta^*_{ii}] \qquad (5)$$

where $\bar{Q}^{*0}$ is a determinant of virial coefficients independent of unit charge of monomeric species. Therefore

$$\bar{Q}^{*0} = \frac{1}{C^*}[C^*_1{}^2\beta^*_{11} + C^*_2{}^2\beta^*_{22} + C^*_3{}^2\beta^*_{33} + \ldots + C^*_n{}^2\beta^*_{nn} + \ldots]$$

$$\bar{Q}^{*0} = \frac{C^*_1{}^2}{C^*}\left[\beta^*_{11} + \frac{C^*_2{}^2}{C^*_1{}^2}\beta^*_{22} + \frac{C^*_3{}^2}{C^*_1{}^2}\beta^*_{33} + \ldots + \frac{C^*_n{}^2}{C^*_1{}^2}\beta^*_{nn} + \ldots\right]$$

From eq 4c

$$\frac{\bar{Q}^{*0}}{x^*_1{}^2} = C^*[\beta^*_{11} + 4k^2{}_2[\mathrm{A}_1]\beta^*_{22} + 9k_2{}^2k_3{}^2[\mathrm{A}_1]^4\beta^*_{33} + 16k_2{}^2k_3{}^2k_4{}^2[\mathrm{A}_1]^6\beta^*_{44} + 25k_2{}^2k_3{}^2k_4{}^2k_2[\mathrm{A}_1]^8\beta^*_{55} + \ldots] \qquad (5a)$$

Equation 5a is also obtainable from eq 4d when the equation is considered independent of unit charge of the effective monomer.

Equations 4d and 5a are now applicable to all reversibly associating systems in which the monomer participates in the reaction leading to the formation of the higher polymers which subsequently act as the effective monomer.

The Determination of $\bar{Q}^*$ or $\bar{Q}^{*0}$ in Nonideal Systems. The determination of the virial coefficients from $\bar{Q}^*$ may be accomplished by stepwise polynomial regression analysis, as previously described (P. W. Chun, M. Fried, and K. S. Yee, submitted for publication).

$$\frac{(\bar{M}_w{}^0)^*_{\mathrm{app}}}{M^*_1} = \bar{\alpha}^*_\omega{}^0$$

and

$$\ln\frac{x^*_1}{x^*_{1,0}} = \int_{C^*_0}^{C^*}\frac{\bar{\alpha}^*_\omega{}^{0-1} - 1}{C^*}\mathrm{d}C^*$$

are readily determined from sedimentation equilibrium measurements. The term $\bar{\alpha}^*_\omega{}^0/x^*_1$ (see eq d of Appendix) must be evaluated prior to the determination of $\bar{Q}^*$. It is noteworthy to mention that $\bar{\alpha}^*_\omega{}^0$ is not equivalent to $\bar{\alpha}_\omega$, the asterisk indicating a unit charge which must be considered, *i.e.*, $\bar{\alpha}^*_\omega{}^0 = \bar{\alpha}_\omega + \mathrm{f}(Z)$. Comparison of eq b and d in the Appendix shows a variation of 2.7% in the k values when the f(Z) function is not included, as confirmed in our previous work. Knowing $\bar{\alpha}^*_\omega$

$$\bar{Q}^* \text{ or } \bar{Q}^{*0} \Rightarrow (\Delta\bar{M}_w)^*_{\mathrm{app}} = (\bar{M}_w)^*_{\mathrm{app}} - (\bar{M}_w{}^0)^*_{\mathrm{app}}$$

$$\frac{(\Delta\bar{M}_w)^*_{\mathrm{app}}}{M^*_1} = \bar{\alpha}^*_\omega - \bar{\alpha}^*_\omega{}^0 = (\Delta\bar{\alpha}^*_\omega)$$

or

$$\frac{(\Delta\bar{M}_w)^{*0}}{M^*_1} = (\Delta\bar{\alpha}_\omega)^{*0}$$

$\bar{Q}^{*0} \neq \bar{Q}^*$, as described in the text; however, $\bar{Q}^{*0}$ may be obtained in a similar fashion as above, providing it is considered independent of unit charge as affected by monomeric species. Theoretical differences obtained in the virial coefficient of $\bar{Q}^{*0}$ and $\bar{Q}^*$ must be further verified by experimental data.

Thus $[\bar{Q}^*/x^*_1{}^2C^*]$ or $[\bar{Q}^{*0}/x^*_1{}^2C^*]$ and the virial coefficients from eq 4d or 5a may be accurately evaluated by stepwise polynomial regression analysis with or without consideration of the effect of unit charge.

Appendix (based on P. W. Chun, M. Fried, and K. S. Yee, submitted for publication)

Reversibly associating protein systems: (1) Steiner's (1952) equation

$$[\mathrm{A}_2] = k_2[\mathrm{A}_1]^2$$

$$[\mathrm{A}_3] = k_2k_3[\mathrm{A}_1]^3$$

$$[\mathrm{A}_4] = k_4k_3k_2[\mathrm{A}_1]^4$$

$$[\mathrm{A}_i] = ik_i\ldots k_4k_3k_2[\mathrm{A}_1]^i$$

$$(\bar{M}_w)_{\mathrm{app}} = \frac{1}{C}[[\mathrm{A}_1] + 4k_2[\mathrm{A}_1]^2 + 9k_2k_3[\mathrm{A}_1]^3 + 16k_2k_3k_4[\mathrm{A}_1]^4 + \ldots] \qquad (a)$$

(C/m) as a function of $[\mathrm{A}_1] = (x_1C/m)$, where C is total weight of monomer and m is the molecular weight of monomer. $\bar{\alpha}_\omega = \bar{M}_w/m$ and $\bar{\alpha}_\omega$ as a function of C

$$\frac{\bar{\alpha}_\omega}{x_1} = [1 + 4k_2[\mathrm{A}_1] + 9k_2k_3[\mathrm{A}_1]^2 + 16k_2k_3k_4[\mathrm{A}_1]^4 + \ldots] \qquad (b)$$

where x_1 – weight fraction of monomer at concentration C (ideal system).

(2) Casassa and Eisenberg definition and reversibly associating systems (only the effect of unit charge on monomer species is considered here, assuming $\beta_{ii} = 0$).

$$M^*{}_i L^*{}_i \left[\frac{\mathrm{d}\ln C^*{}_i/M^*{}_i}{\mathrm{d}(x^2)}\right]^{-1} = 1 + \frac{C^*{}_i}{M^*{}_i}\left[\frac{\nu^*{}_{i+}{}^2}{m_+} + \frac{\nu^*{}_{i-}{}^2}{m_-}\right]$$

where $L^*{}_i = (1 - \bar{v}^*\rho)\omega^2/2RT$. $\nu^*{}_{2\pm}$ is the number of moles of ionic species. Assume $L^*{}_1 = L^*{}_2 = L^*{}_3 \ldots L^*{}_n$; $C^*{}_1$, $C^*{}_2$, $C^*{}_3 \ldots$ are the weight concentrations of the *n*-mers, respectively, and the total concentration of C^* is given by $C^* = C^*{}_1 + C^*{}_2 + C^*{}_3 + \ldots$ or $\mathrm{d}C^* = \mathrm{d}C^*{}_1 + \mathrm{d}C^*{}_2 + \mathrm{d}C^*{}_3 + \ldots$.

$$(\bar{M}_{\mathrm{w}}{}^0)^*{}_{\mathrm{app}} = \frac{1}{C^*}\sum_i\left[\frac{M^*{}_i C^*{}_i}{1 + iC^*{}_i\left(\frac{X}{M^*{}_i}\right)}\right] \cong \frac{1}{C^*}\sum_i M^*{}_i C^*{}_i\left[1 - iC^*{}_i\left(\frac{X}{M^*{}_i}\right)\right] \quad \text{(c)}$$

where

$$C^*{}_i\left(\frac{X}{M^*{}_i}\right) \ll 1$$

By binomial approximation

$$\frac{\bar{\alpha}^*{}_\omega{}^0}{x^*{}_1} = [(1 - [\mathrm{A}_1]X) + 4k_2[\mathrm{A}_1](1 - 4k_2[\mathrm{A}_1]^2X) + 9k_2k_3[\mathrm{A}_1]^2(1 - 9k_2k_3[\mathrm{A}_1]^3X) + \ldots] \quad \text{(d)}$$

(3) Jeffrey and Coates' (1962) equation.

Assuming the species in equilibrium were even numbered (this is the case for insulin at low pH), that is, the monomer as such participates only in the reaction leading to the formation of the dimer which subsequently acts as the effective monomer, when $i = 1, 2, 4, 6 \ldots$

$$\frac{\bar{\alpha}^*{}_\omega{}^0}{x^*{}_1} = [1 + [\mathrm{A}_1](4k_2 - X) + [\mathrm{A}_1]^3(16k_4k_2{}^2 - k_4k_2X) + [\mathrm{A}_1]^5(36k_6k_4k_2{}^3 - 36k_5k_4k_2{}^2X) + \ldots] \quad \text{(e)}$$

(4) Evaluation of equilibrium constants from polynomials derived for odd-numbered series, when $i = 1, 3, 5, 7 \ldots$.

$$\frac{\bar{\alpha}^*{}_\omega{}^0}{x^*{}_1} = [(1 - [\mathrm{A}_1]X) + 9k_3[\mathrm{A}_1]^2(1 - 9k_3[\mathrm{A}_1]^3X) + 25k_5k_3[\mathrm{A}_1]^4(1 - 25k_5k_3[\mathrm{A}_1]^5X) + \ldots] \quad \text{(f)}$$

When $i = 1, 2, 3, 5, 7 \ldots$.

$$\frac{\bar{\alpha}^*{}_\omega{}^0}{x^*{}_1} = [(1 - [\mathrm{A}_1]X) + 9k_2k_3[\mathrm{A}_1]^2(1 - 9k_2k_3[\mathrm{A}_1]^3X) + 25k_5k_3k_2{}^2[\mathrm{A}_1]^4(1 - 25k_5k_3k_2{}^2[\mathrm{A}_1]^5X] + \ldots] \quad \text{(g)}$$

Equations f and g describe two systems differing in the mode of association required to form the odd-numbered polymers in equilibrium, one being based on an effective monomer and the other on an effective dimer. These derivations are theoretical and have not yet been applied to a reversibly associating protein system. As described in the text, $\bar{\alpha}_\omega/x_1 \neq \bar{\alpha}^*{}_\omega{}^0/x^*{}_1$. In the Steiner equation $[\mathrm{A}_1] = (x_1C/m)$, whereas $[\mathrm{A}_1] = (x^*{}_1C^*/M^*{}_1)$ in the Casassa and Eisenberg (1960, 1961) definition.

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