Analysis of a Nonideal Associating System Involving Two Different Monomer Units by Colligative Methods*

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ABSTRACT: A general method is developed for the analysis of nonideal mixed associating systems from colligative data with the objective of determining the consecutive association constants. The method depends on the use of curves of number-average molecular weight as a function of concentration for a series of compositions. From these the apparent concentrations of monomer units are obtained, which are dependent on the magnitude of the virial coefficients. For a series of systems of increasing generality equations are developed which may be solved numerically for the virial coefficients, permitting evaluation of the true monomer concentrations and, from these, the actual association constants.

In two earlier publications a general method has been presented for obtaining the consecutive association constants from colligative data for a generalized associating system containing two different molecular species, which could undergo both self-association and association with each other (Steiner, 1968, 1970). While no restrictions were introduced as to the nature of the complex species or the mode of association, ideal behavior was assumed for all species. It is the purpose of the present paper to eliminate the restriction to ideal systems and to extend the theoretical treatment to real systems. The treatment here will be confined to the case of two associating species (Steiner, 1968). The nonideal general case (Steiner, 1970) will be presented separately.

The system is postulated to contain two different monomer units, A and B, either or both of which may self-associate, as well as form mixed complex species. The generalized mode is described by the following equations

$$
\begin{array}{lll}
\mathrm{A} & + \mathrm{A} \rightleftharpoons \mathrm{A_2};\ K_{20} = [\mathrm{A_2}]/[\mathrm{A}]^2 & \\
\mathrm{A_2} & + \mathrm{A} \rightleftharpoons \mathrm{A_3};\ K_{30} = [\mathrm{A_3}]/[\mathrm{A}]^3 & \\
\vdots & & \\
\mathrm{A}_{i-1} & + \mathrm{A} \rightleftharpoons \mathrm{A}_i;\ K_{i0} = [\mathrm{A}_i]/[\mathrm{A}]^i & \\
\mathrm{B} & + \mathrm{B} \rightleftharpoons \mathrm{B_2};\ K_{02} = [\mathrm{B_2}]/[\mathrm{B}]^2 & \\
\mathrm{B_2} & + \mathrm{B} \rightleftharpoons \mathrm{B_3};\ K_{03} = [\mathrm{B_3}]/[\mathrm{B}]^3 & (1) \\
\vdots & & \\
\mathrm{B}_{j-1} & + \mathrm{B} \rightleftharpoons \mathrm{B}_j;\ K_{0j} = [\mathrm{B}_j]/[\mathrm{B}]^j & \\
\mathrm{A} & + \mathrm{B} \rightleftharpoons \mathrm{AB};\ K_{11} = [\mathrm{AB}]/[\mathrm{A}][\mathrm{B}] & \\
\mathrm{AB} & + \mathrm{B} \rightleftharpoons \mathrm{AB_2};\ K_{12} = [\mathrm{AB_2}]/[\mathrm{A}][\mathrm{B}]^2 & \\
\mathrm{AB} & + \mathrm{A} \rightleftharpoons \mathrm{A_2B};\ K_{21} = [\mathrm{A_2B}]/[\mathrm{A}]^2[\mathrm{B}] & \\
\mathrm{A}_i\mathrm{B}_{j-1} & + \mathrm{B} \rightleftharpoons \mathrm{A}_i\mathrm{B}_j;\ K_{ij} = [\mathrm{A}_i\mathrm{B}_j]/[\mathrm{A}]^i[\mathrm{B}]^j &
\end{array}
$$

where [A] and [B] are the molar concentrations of free monomeric A and B, respectively.

Examples of heterogeneous associating systems are very common in biochemistry, although quantitative studies are few. Some random examples are the antigen–antibody reaction, the hemoglobin–haptoglobin reaction, the combination of trypsin with protein inhibitors, and the association of purine and pyrimidine nucleosides.

The theory to be described here is applicable to any colligative method. Membrane osmometry is ordinarily the method of choice for molecules of mol wt $>10^3$, while vapor pressure depression may be used for small molecules.

Results

We shall cite in this section the principal results of the theoretical development, leaving the details of the derivation to the Appendix. It will be assumed that the deviation from ideality of the real system can be accounted for in terms of three second virial coefficients, two of which, B_{AA} and B_{BB}, reflect the self-interaction of species A and B, respectively, while the third, B_{AB}, originates from the mixed interaction (apart from molecular association) of species A with species B. Details are given in the Appendix. In practice, this assumption is likely to be valid for the usual experimental conditions chosen for studies of protein solutions (concentrations less than 2%, ionic strength 0.1 or greater).

In terms of the above model, the relationship between the observed osmotic pressure, π, and the *total* weight concentrations (in grams per kilogram of solvent), $c_{\mathrm{A,t}}$ and $c_{\mathrm{B,t}}$, of species A and B assumes the form

$$\frac{\pi V_{\mathrm{m}}}{RT} = \frac{c}{M_{\mathrm{n}}} + \frac{B_{\mathrm{AA}}}{2}c_{\mathrm{A,t}}^2 + \frac{B_{\mathrm{BB}}}{2}c_{\mathrm{B,t}}^2 + \frac{B_{\mathrm{AB}}}{2}c_{\mathrm{A,t}}c_{\mathrm{B,t}} \equiv m^* \quad (2)$$

where V_{m} is the volume (in milliliters) containing 1 kg of solvent, M_{n} is the number-average molecular weight, c $(= c_{\mathrm{A,t}} + c_{\mathrm{B,t}})$ is the total concentration, R is the gas constant, T is the absolute temperature, and m^* is the apparent molality of the system.

The quantities B_{AA} and B_{BB} may ordinarily be determined from independent measurements upon solutions of pure A and pure B (see Appendix). If the corresponding terms are

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subtracted from eq 2, we may define a new quantity, m^{**}, such that

$$\begin{aligned} m^{**} &\equiv \frac{\pi V_m}{RT} - \frac{B_{AA}}{2}c_{A,t}^2 - \frac{B_{BB}}{2}c_{B,t}^2 \\ &= m^* - \frac{B_{AA}}{2}c_{A,t}^2 - \frac{B_{BB}}{2}c_{B,t}^2 \qquad (3) \\ &= \frac{c}{M_n} + \frac{B_{AB}}{2}c_{A,t}c_{B,t} \end{aligned}$$

The difference between m^{**} and m is due wholly to interactions between species A and species B.

For the ideal case, where $B_{AA} = B_{BB} = B_{AB} = 0$, we have

$$\frac{\pi V_m}{RT} = \frac{c}{M_n} = m \qquad (4)$$

where m is the actual molality of the system and is equal to m^{**}.

In an earlier publication (Steiner, 1968) it was shown that, for the ideal case

$$\ln Z_A + \beta \ln Z_B = \int (\alpha_n^{-1} - 1)\frac{dm}{m} + \ln\{1/(1+\beta)\} + \beta \ln\{\beta/(1+\beta)\} \equiv \Phi \qquad (5)$$

Here, Z_A and Z_B are the mole fractions of free monomeric A and free monomeric B, respectively, and are given by

$$\begin{aligned} Z_A &= [A]/m \\ Z_B &= [B]/m \end{aligned} \qquad (6)$$

β is the ratio of the *total* molality of B, $m_{B,t}$, to the *total* molality of A, $m_{A,t}$; *i.e.*, $\beta = m_{B,t}/m_{A,t}$, α_n is the number-average degree of association and is equal to $(m_{A,t} + m_{B,t})/m$, or to M_n/M_0, where M_0 is the number-average molecular weight predicted for the nonassociated system where only monomers are present and is given by

$$M_0 = M_A m_{A,t}/m_t + M_B m_{B,t}/m_t \qquad (7)$$

where M_A and M_B are the molecular weights of monomeric A and B, respectively, and $m_t(= m_{A,t} + m_{B,t})$ is the *total* molality of monomeric units of both species, whether free or associated.

A second equation may also be derived which yields essentially the same information as eq 5 (see Appendix).

$$\ln X_A + \beta \ln X_B = (1+\beta)(\alpha_n^{-1} - 1) + (1+\beta)\int_0^{m_t} (\alpha_n^{-1} - 1)\frac{dm_t}{m_t} + \ln\{1/(1+\beta)\} + \beta \ln\{\beta/(1+\beta)\} \qquad (8)$$

where $X_A = [A]/m_t$ and $X_B = [B]/m_t$. For the nonideal case we have in place of eq 8 (see Appendix)

$$\ln X_A^{**} + \beta \ln X_B^{**} = (1+\beta)(\alpha_n^{**} - 1) + (1+\beta)\int_0^{m_t} (\alpha_n^{**} - 1)\frac{dm_t}{m_t} + \ln\{1/(1+\beta)\} + \beta \ln\{\beta/(1+\beta)\} \qquad (9)$$

Here α_n^{**} $(= m_t/m^{**})$ is the apparent number-average degree of association and X_A^{**} and X_B^{**} are the apparent values of X_A and X_B, respectively.

We also have for ideal systems, from the previous paper (Steiner, 1968)

$$\ln Z_B = \left(\frac{d\Phi}{d\beta}\right)_m \qquad (10)$$

where Φ is given by eq 5. For nonideal systems, we have in this case the parallel equation (see Appendix)

$$\ln Z_B^{**} = \left(\frac{d\Phi^{**}}{d\beta}\right)_{m^{**}} \qquad (11)$$

Here Φ^{**} is the nonideal analog of Φ and is given by

$$\begin{aligned} \Phi^{**}(\beta, m^{**}) &= \ln Z_A^{**} + \beta \ln Z_B^{**} \\ &= (1+\beta)\int_0^{m^{**}} (\alpha_n^{**-1} - 1)\frac{dm^{**}}{m^{**}} + \qquad (12) \\ &\quad \ln\{1/(1+\beta)\} + \beta \ln\{\beta/(1+\beta)\} \end{aligned}$$

The quantity $\ln Z_B^{**}$ is equal to the slope of Φ^{**} as a function of β at constant m^{**} and may be evaluated by the same procedures as for the ideal case (Steiner, 1968). The quantities Z_B^{**} and X_B^{**} are related by

$$Z_B^{**} = X_B^{**}\frac{m_t}{m^{**}} \qquad (13)$$

If X_B^{**} is known, then X_A^{**} may be computed from eq 9. It may also be shown (see Appendix) that

$$\begin{aligned} [A^{**}] &= X_A^{**} m_t \\ &= [A]\exp(B_{AB}M_A M_B m_{B,t}/2) \end{aligned} \qquad (14)$$

and

$$\begin{aligned} [B^{**}] &= X_B^{**} m_t \\ &= [B]\exp(B_{AB}M_A M_B m_{A,t}/2) \end{aligned} \qquad (15)$$

The apparent values, $[A^{**}]$ and $[B^{**}]$, of the concentrations of free monomer units are related to the true values by eq 14 and 15. In order to convert the apparent values of [A] and [B] to the actual values, a knowledge of B_{AB} is required. Once [A] and [B] are known, the consecutive association constants may be computed using the methods described in the preceding paper (Steiner, 1968).

We shall next discuss the actual evaluation of B_{AB}.

It is most convenient to consider a series of cases of increasing generality.

$$\mathrm{A} + \mathrm{B} \leftrightarrows \mathrm{AB} \tag{I}$$

A and B do not self-associate; AB is the only mixed species.

$$m_{\mathrm{t}} = [\mathrm{A}] + [\mathrm{B}] + 2K_{11}[\mathrm{A}][\mathrm{B}] \tag{16}$$

and, from eq A-37

$$X_{\mathrm{A}}^{**}(X_{\mathrm{B}}^{**})^{\beta} = X_{\mathrm{A}}X_{\mathrm{B}}^{\beta} \exp(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}) \tag{17}$$

When $\beta = 1$, $X_{\mathrm{A}} = X_{\mathrm{B}}$, $X_{\mathrm{A}}^{**} = X_{\mathrm{B}}^{**}$, and

$$X_{\mathrm{A}}^{**} = X_{\mathrm{A}} \exp(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}/2) = X_{\mathrm{B}}^{**} = X_{\mathrm{B}} \exp(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}/2) \tag{18}$$

$$[\mathrm{A}] = [\mathrm{B}] = [\mathrm{A}^{**}] \exp(-B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{A}}m_{\mathrm{B,t}}/2)$$

Also

$$m_{\mathrm{t}} = 2[\mathrm{A}^{**}] \exp(-B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}/2) + 2K_{11}[\mathrm{A}^{**}]^2 \exp(-B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}) \tag{19}$$

$$\frac{m_{\mathrm{t}}}{2[\mathrm{A}^{**}]} \exp(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}/2) - K_{11}[\mathrm{A}^{**}] \exp(-B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}/2) = 1 \tag{20}$$

Since all the quantities in eq 20 are known except for B_{AB}, this equation may be solved numerically for B_{AB}, when $\beta = 1$.

$$\begin{array}{lcl} \mathrm{A} & + \mathrm{B} \rightleftarrows & \mathrm{AB} \\ \mathrm{AB} & + \mathrm{B} \rightleftarrows & \mathrm{AB}_2 \\ \vdots & & \\ \mathrm{AB}_{i-1} & + \mathrm{B} \rightleftarrows & \mathrm{AB}_i \end{array} \tag{II}$$

(A and B do not self-associate; no more than one molecule of A is present in any mixed species.)

In this case

$$m^{**} = [\mathrm{A}] + [\mathrm{B}] + [\mathrm{A}]\sum_j K_{1j}[\mathrm{B}]^j + \frac{1}{2\beta}B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}^2 \tag{21}$$

$$m_{\mathrm{A,t}} = [\mathrm{A}] + [\mathrm{A}]\sum_j K_{1j}[\mathrm{B}]^j \tag{22}$$

and

$$\begin{aligned} m^{**} - m_{\mathrm{A,t}} &= [\mathrm{B}] + \frac{1}{2\beta}B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}^2 \\ &= [\mathrm{B}^{**}] \exp(-B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{A,t}}/2) + \frac{1}{2\beta}B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}^2 \end{aligned} \tag{23}$$

Equation 23 may be solved numerically for B_{AB}, the only unknown. Case I may of course be regarded as a special instance of case II.

$$\begin{array}{lcl} \mathrm{A} + \mathrm{A} & \rightleftarrows & \mathrm{A}_2 \\ \mathrm{A} + \mathrm{A}_2 & \rightleftarrows & \mathrm{A}_3 \\ \vdots & & \\ \mathrm{A} + \mathrm{A}_{i-1} & \rightleftarrows & \mathrm{A}_i \\ \mathrm{B} + \mathrm{B} & \rightleftarrows & \mathrm{B}_2 \\ \mathrm{B} + \mathrm{B}_2 & \rightleftarrows & \mathrm{B}_3 \\ \vdots & & \\ \mathrm{B} + \mathrm{B}_{i-1} & \rightleftarrows & \mathrm{B}_i \\ \mathrm{A} + \mathrm{B} & \rightleftarrows & \mathrm{AB} \end{array} \tag{III}$$

(A and B undergo self-association; AB is the only mixed complex formed.)

The set of association constants characterizing the self-association of A and B may be obtained by independent measurements upon pure A and B. One then has

$$m_{\mathrm{A,t}} = \Sigma iK_{i0}[\mathrm{A}]^i + K_{11}[\mathrm{A}][\mathrm{B}]$$

$$m_{\mathrm{B,t}} = \Sigma jK_{0j}[\mathrm{B}]^j + K_{11}[\mathrm{A}][\mathrm{B}]$$

$$\begin{aligned} m_{\mathrm{A,t}} - m_{\mathrm{B,t}} &= \Sigma iK_{i0}[\mathrm{A}]^i - \Sigma jK_{0j}[\mathrm{B}]^j \\ &= \Sigma iK_{i0}[\mathrm{A}^{**}]^i \exp(-iB_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}/2) - \\ &\quad -\Sigma jK_{0j}[\mathrm{B}^{**}]^j \exp(-\ jB_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{A,t}}/2) \end{aligned} \tag{24}$$

Since the set of K_{i0} and K_{0j} are known, B_{AB} is the only unknown in eq 24, which may be solved numerically.

$$\begin{array}{lcl} \mathrm{A} & + \mathrm{A} \rightleftarrows & \mathrm{A}_2 \\ \mathrm{A} & + \mathrm{A}_2 \rightleftarrows & \mathrm{A}_3 \\ \vdots & & \\ \mathrm{A} & + \mathrm{A}_{i-1} \rightleftarrows & \mathrm{A}_i \\ \mathrm{B} & + \mathrm{B} \rightleftarrows & \mathrm{B}_2 \\ \mathrm{B} & + \mathrm{B}_2 \rightleftarrows & \mathrm{B}_3 \\ \vdots & & \\ \mathrm{B} & + \mathrm{B}_{i-1} \rightleftarrows & \mathrm{B}_i \\ \mathrm{A} & + \mathrm{B} \rightleftarrows & \mathrm{AB} \\ \mathrm{AB} & + \mathrm{B} \rightleftarrows & \mathrm{AB}_2 \\ \vdots & & \\ \mathrm{AB}_{j-1} & + \mathrm{B} \rightleftarrows & \mathrm{AB}_j \end{array} \tag{IV}$$

(A and B undergo self-association; no more than one molecule of A is present in any mixed complex.)

We now have

$$m^{**} = \Sigma K_{i0}[\mathrm{A}]^i + \Sigma K_{0j}[\mathrm{B}]^j + [\mathrm{A}]\Sigma K_{ij}[\mathrm{B}]^j + \frac{1}{2\beta}B_{\mathrm{AB}}m_{\mathrm{B,t}}^2M_{\mathrm{A}}M_{\mathrm{B}} \tag{25}$$

$$m_{\mathrm{A,t}} = \Sigma iK_{i0}[\mathrm{A}]^i + [\mathrm{A}]\Sigma K_{ij}[\mathrm{B}]^j \tag{26}$$

and

$$\begin{aligned} m_{\mathrm{A,t}} - m^{**} &= -\Sigma K_{i0}[\mathrm{A}]^i + \Sigma iK_{i0}[\mathrm{A}]^i - \frac{1}{2\beta}B_{\mathrm{AB}}m_{\mathrm{B,t}}^2M_{\mathrm{A}}M_{\mathrm{B}} - \Sigma K_{0j}[\mathrm{B}]^j = \Sigma(i - 1)K_{i0}[A^{**}]^i \times \\ &\quad \exp(-iB_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}/2) - \Sigma K_{0j}[B^{**}]^j \times \\ &\quad \exp(-jB_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{A,t}}/2) - \frac{1}{2\beta}B_{\mathrm{AB}}m_{\mathrm{B,t}}^2M_{\mathrm{A}}M_{\mathrm{B}} \end{aligned} \tag{27}$$

If the set of K_{i0} and K_{0j} which characterize the self-association of A and B are determined from independent measurements upon pure A and B, B_{AB} is the only unknown in eq 27, which may be solved numerically.

$$\begin{array}{l} \mathrm{A} + \mathrm{B} \rightleftarrows \mathrm{AB} \\ \mathrm{A} + \mathrm{AB} \rightleftarrows \mathrm{A_2B} \\ \mathrm{AB} + \mathrm{B} \rightleftarrows \mathrm{AB_2} \end{array} \qquad \text{(V)}$$

(A and B do not self-associate; AB, A_2B, and AB_2 are the only mixed complex species.) In this case we have

$$m = [\mathrm{A}] + [\mathrm{B}] + [\mathrm{AB}] + [\mathrm{A_2B}] + [\mathrm{AB_2}] \qquad (28)$$

$$m_{\mathrm{A,t}} = [\mathrm{A}] + [\mathrm{AB}] + 2[\mathrm{A_2B}] + [\mathrm{AB_2}] \qquad (29)$$

$$m_{\mathrm{B,t}} = [\mathrm{B}] + [\mathrm{AB}] + [\mathrm{A_2B}] + 2[\mathrm{AB_2}] \qquad (30)$$

This set of three simultaneous equations may be used to eliminate $[AB_2]$ and $[A_2B]$.

$$\begin{aligned} 3m - m_{\mathrm{t}} &= 2[\mathrm{A}] + 2[\mathrm{B}] + [\mathrm{AB}] \\ &= 2[\mathrm{A}] + 2[\mathrm{B}] + K_{11}[\mathrm{A}][\mathrm{B}] \end{aligned} \qquad (31)$$

We also have

$$\begin{aligned} m_{\mathrm{A,t}} &= [\mathrm{A}] + K_{11}[\mathrm{A}][\mathrm{B}] + \ldots \\ &= [\mathrm{A^{**}}] \exp -[(^1/_2)(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}})] + \\ &\quad K_{11}[\mathrm{A^{**}}][\mathrm{B^{**}}] \exp -[(^1/_2)(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{t}})] + \ldots \qquad (32) \\ &= [\mathrm{A^{**}}](1 - (^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}} + \ldots) + \\ &\quad K_{11}[\mathrm{A^{**}}][\mathrm{B^{**}}] + \ldots \end{aligned}$$

and

$$\frac{m_{\mathrm{A,t}} - [\mathrm{A^{**}}]}{[\mathrm{A^{**}}][\mathrm{B^{**}}]} = K_{11} - (^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}\frac{m_{\mathrm{B,t}}}{[\mathrm{B^{**}}]} + \qquad (33)$$

Upon extrapolation of the left-hand side of eq 33 at constant β to $[A^{**}] = 0$ and $[B^{**}] = 0$

$$\lim_{\substack{[\mathrm{A^{**}}] \to 0 \\ [\mathrm{B^{**}}] \to 0}} \left\{\frac{m_{\mathrm{A,t}} - [\mathrm{A^{**}}]}{[\mathrm{A^{**}}][\mathrm{B^{**}}]}\right\} = K_{11} - (^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}} \equiv \sigma_{11} \qquad (34)$$

Equation 31 may now be rewritten

$$\begin{aligned} 3m^{**} - \frac{3}{2\beta}B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}{}^2 - m_{\mathrm{t}} &= 2[\mathrm{A^{**}}] \times \\ \exp -[(^1/_2)(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}})] &+ 2[\mathrm{B^{**}}] \times \\ \exp -[(^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{A,t}})] + (\sigma_{11} &+ (^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}) \times \\ [\mathrm{A^{**}}][\mathrm{B^{**}}] \exp - [(^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{t}})] & \qquad (35) \end{aligned}$$

If σ_{11} is obtained by extrapolation, using eq 34, then B_{AB} is the only unknown in eq 35, which may now be solved numerically.

$$\begin{array}{l} \mathrm{A} + \mathrm{A} \rightleftarrows \mathrm{A_2} \\ \mathrm{A} + \mathrm{A_2} \rightleftarrows \mathrm{A_3} \\ \vdots \\ \mathrm{A}_{i-1} + \mathrm{A} \rightleftarrows \mathrm{A}_i \\ \mathrm{B} + \mathrm{B} \rightleftarrows \mathrm{B_2} \\ \mathrm{B} + \mathrm{B_2} \rightleftarrows \mathrm{B_3} \\ \vdots \\ \mathrm{B}_{i-1} + \mathrm{B} \rightleftarrows \mathrm{B}_i \\ \mathrm{A} + \mathrm{B} \rightleftarrows \mathrm{AB} \\ \mathrm{AB} + \mathrm{B} \rightleftarrows \mathrm{AB_2} \\ \mathrm{AB} + \mathrm{A} \rightleftarrows \mathrm{A_2B} \end{array} \qquad \text{(VI)}$$

(A and B undergo self-association; AB, A_2B, and AB_2 are the only mixed complex species present.) In this case, eq 35 is replaced by

$$\begin{aligned} 3m^{**} - \frac{3}{2\beta}B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}}{}^2 - m_{\mathrm{t}} &= 3\Sigma K_{i0}[\mathrm{A^{**}}]^i \times \\ \exp\{-(i/2)(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}})\} &- \Sigma iK_{i0}[\mathrm{A^{**}}]^i \times \\ \exp\{-(\mathrm{i}/2)(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{B,t}})\} &+ 3\Sigma K_{0j}[\mathrm{B^{**}}]^j \times \\ \exp\{-(/2)(jB_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{A,t}})\} &- \Sigma jK_{0j}\,[\mathrm{B^{**}}]^j \times \\ \exp\{-(j/2)(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{A,t}})\} + (\sigma_{11} &+ (^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}) \times \\ [\mathrm{A^{**}}][\mathrm{B^{**}}] \exp\{-(^1/_2)(B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}m_{\mathrm{t}})\} & \qquad (36) \end{aligned}$$

If the set of K_{i0} and K_{0j}, which characterize the self-association of A and B, are determined from independent measurements upon pure A and B, B_{AB} is the only unknown in eq 36, which may be solved numerically. The parameter σ_{11} may in this case be determined from

$$\lim_{[\mathrm{A^{**}}],[\mathrm{B^{**}}] \to 0} \left\{\frac{m_{\mathrm{t}} - [\mathrm{A^{**}}] - [\mathrm{B^{**}}]}{[\mathrm{A^{**}}][\mathrm{B^{**}}]}\right\} = 2\sigma_{11} + \frac{2}{\beta}K_{20} + 2\beta K_{02} \qquad (37)$$

The completely general case is that described by eq 1 and corresponds to the absence of all restrictions upon both the self-association of A and B and the formation of mixed complexes. No analytical solution has been obtained for this case. One has available however a set of equations of the type

$$\begin{aligned} m_{\mathrm{t},1} &= \Sigma(i + j)K_{ij}[\mathrm{A_1^{**}}]^i[\mathrm{B_1^{**}}]^j \times \\ &\qquad \exp\{-(^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}(im_{\mathrm{B,t,1}} + jm_{\mathrm{A,t,1}})\} \\ m_{\mathrm{t},2} &= \Sigma(i + j)K_{ij}[\mathrm{A_2^{**}}]^i[\mathrm{B_2^{**}}]^j \times \\ &\qquad \exp\{-(^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}(im_{\mathrm{B,t,2}} + jm_{\mathrm{A,t,2}})\} \qquad (38) \\ m_{\mathrm{t},n} &= \Sigma(i + k)K_{ij}[\mathrm{A}_n{}^{**}]^i[\mathrm{B}_n{}^{**}]^j \times \\ &\qquad \exp\{-(^1/_2)B_{\mathrm{AB}}M_{\mathrm{A}}M_{\mathrm{B}}(im_{\mathrm{B,t},n} + jm_{\mathrm{A,t},n})\} \end{aligned}$$

Here the subscripts 1, 2, . . . , n correspond to different concentrations for the same value of β. If a sufficient number of concentrations are measured, the set of K_{ij} and B_{AB} may be regarded as "overdetermined." Van Holde *et al.* (1969) have described computational methods for evaluating the unknowns in this case.

Discussion

The preceding sections have described the extension of the theory developed earlier for ideal systems (Steiner, 1968) to real systems. The present treatment is applicable provided that three virial coefficients are adequate to account for the deviation from ideality of the system under consideration.

The computational problems encountered in obtaining the apparent quantities [A**] and [B**] from actual data are similar to those encountered for the ideal case and have been discussed in the earlier paper (Steiner, 1968). Probably the most serious source of potential error is in the evaluation of the slope, using eq 36. Perhaps the most satisfactory way of achieving this is by a polynomial fit of Φ as a function of β using a suitable computer program, such as POLFIT from the General Electric program library.

While it has not been possible to obtain a general analytic solution capable of permitting evaluation of B_{AB} for an unrestricted associating system, the special cases considered here should suffice for the interpretation of many real systems. For the most general case the computational methods described by Van Holde *et al.* (1969) may be applicable.

The analysis of mixed systems places stringent requirements upon the data, particularly when self-association of the monomeric species is present. Realization of the full potentialities of the method must probably await improvements in instrumentation.

Many systems of interest are actually charged polyelectrolytes. An argument completely analogous to that presented by Adams (1965a,b) indicates that the theory may be used in unchanged form for this case, provided that components are defined as specified by Casassa and Eisenberg (1960, 1964).

Appendix

We shall first develop the theory explicitly for the case of two uncharged macromolecules which undergo both self-association and association with each other. As will be seen later, the results can be extended without change to the case of charged macromolecular species, provided that a suitable definition of components is made.

There are two thermodynamic components, A and B, in this system. If terms in the virial expansion higher than the second power in concentration are neglected, then the osmotic pressure for a mixed system may be expressed by, for dilute solutions (Casassa and Eisenberg, 1964):

$$\frac{\pi V_m}{RTc} = \frac{1}{M_n} + c\sum_j\sum_k \beta'_{jk}y_jy_k/M_jM_k \qquad \text{(A-1)}$$

Here π is the osmotic pressure, R is the gas constant, T is the absolute temperature, c is the total concentration of nondiffusible solute in grams per kilogram of solvent, V_m is the volume (in milliliters) containing one kilogram of solvent, M_n is the number-average molecular weight, y_j, y_k are the weight fractions of components j and k, respectively, and M_j, M_k are the monomer molecular weights of components j and k, respectively.

The parameter β'_{jk} is, in the terminology of Casassa and Eisenberg (1964), approximately equal to β_{jk}, which is defined by

$$\beta_{jk} = \frac{\partial \ln \gamma_j}{\partial m_k} \qquad \text{(A-2)}$$

where γ_j is the activity coefficient of component j and m_k is the molality of component k. The minor distinction between β_{jk} and β'_{jk} (Casassa and Eisenberg, 1964) is not important for our purposes here.

Equation A-1 may be rewritten as

$$\frac{\pi V_m}{RT} = \frac{c}{M_n} + \sum_j\sum_k \beta'_{jk}c_{j,t}c_{k,t}/M_jM_k \qquad \text{(A-3)}$$

where $c_{j,t}$, $c_{k,t}$ are the total concentrations of components i and k, respectively. For the present system, where there are only two components, A and B, we have

$$\frac{\pi V_m}{RT} = \frac{c}{M_n} + \beta'_{AA}c_{A,t}^2/M_A^2 + \beta'_{BB}c_{B,t}^2/M_B^2 + 2\beta'_{AB}c_{A,t}c_{B,t}/M_AM_B \equiv m^* = m + (^1/_2)B_{AA}c_{A,t}^2 + (^1/_2)B_{BB}c_{B,t}^2 + (^1/_2)B_{AB}c_{A,t}c_{B,t} \qquad \text{(A-4)}$$

Here m^* is the apparent total molality of the system; m ($= c/M_n$) is the true molality; B_{AA} ($= 2\beta'_{AA}/M_A^2$), B_{BB} ($= 2\beta'_{BB}/M_B^2$), and B_{AB} ($= 4\beta'_{AB}/M_AM_B$) are the virial coefficients characterizing the deviation of the system from ideality.

The apparent total molality, m^*, is the experimentally determined quantity. For a system showing ideal behavior $m^* = m$. It has already been shown that, for the ideal case (Steiner, 1968)

$$m_{A,t} = [A]\frac{\partial m}{\partial [A]}$$

$$m_{B,t} = [B]\frac{\partial m}{\partial [B]} \qquad \text{(A-5)}$$

$$m_t = m_{A,t} + m_{B,t} = [A]\frac{\partial m}{\partial [A]} + [B]\frac{\partial m}{\partial [B]}$$

Here $m_{A,t}$($= c_{A,t}/M_A$) and $m_{B,t}$($= c_{B,t}/M_B$) are the total molal concentrations of A and B monomer units, respectively, whether free or complexed.

It is convenient to define the quantities [A*] and [B*] such that

$$[A^*]\frac{\partial m^*}{\partial [A^*]} = m_{A,t} \qquad \text{(A-6)}$$

$$[B^*]\frac{\partial m^*}{\partial [B^*]} = m_{B,t} \qquad \text{(A-7)}$$

and

$$[A^*]\frac{\partial m^*}{\partial [A^*]} + [B^*]\frac{\partial m^*}{\partial [B^*]} = m_t \qquad \text{(A-8)}$$

The quantities [A*] and [B*] are the apparent values of [A] and [B]. Equation A-8 may be rewritten as

$$(1 + \beta)[A^*]\frac{\partial \ln m^*}{\partial [A^*]} = \alpha_n^* \qquad \text{(A-9)}$$

where $\beta = m_{B,t}/m_{A,t}$; $\alpha_n^* =$ apparent number-average degree of association $= m_t/m^*$.

Integration of eq A-9 yields, if β is maintained constant

$$\ln [A^*] = (1 + \beta) \int \alpha_n^{*-1} \frac{dm^*}{m^*} + G([B^*]) \quad \text{(A-10)}$$

where G is an arbitrary function of [B*].

If one defines the function ψ such that

$$\psi \equiv \ln [A^*] - (1 + \beta) \int \alpha_n^{*-1} \frac{dm^*}{m^*} - G([B^*]) = 0 \quad \text{(A-11)}$$

then

$$[A^*] \frac{\partial \ln m^*}{\partial [A^*]} = \frac{[A^*]}{m^*} \frac{\partial m^*}{\partial [A^*]}$$

$$= -\frac{[A^*]}{m^*} \frac{\partial \psi}{\partial [A^*]} \Big/ \frac{\partial \psi}{\partial m^*} \quad \text{(A-12)}$$

$$= \alpha_n^*/(1 + \beta)$$

and

$$[B^*] \frac{\partial \ln m^*}{\partial [B^*]} = -\frac{\alpha_n^*}{(1 + \beta)} \frac{dG}{d \ln [B^*]} \quad \text{(A-13)}$$

Upon substituting the above into eq A-8

$$\alpha_n^* - \alpha_n^* \Big/ (1 + \beta) + \frac{\alpha_n^*}{(1 + \beta)} \frac{dG}{d \ln [B^*]} = 0 \quad \text{(A-14)}$$

or

$$\frac{dG}{d \ln [B^*]} = -\beta \quad \text{(A-15)}$$

and

$$G = -\beta \ln [B^*] + D \quad \text{(A-16)}$$

where D is a constant.

Finally, we have

$$\ln [A^*] + \beta \ln [B^*] = (1 + \beta) \int \alpha_n^{*-1} \frac{dm^*}{m^*} + D \quad \text{(A-17)}$$

Equation A-17 is the analog for nonideal systems of eq A-18 below, which was derived earlier for ideal systems (Steiner, 1968).

$$\ln [A] + \beta \ln [B] = (1 + \beta) \int \alpha_n^{-1} \frac{dm}{m} + D \quad \text{(A-18)}$$

The integral in the right-hand side of eq A-17 may be rewritten, using eq A-19.

$$\int \alpha_n^{*-1} \frac{dm^*}{m^*} \int = \int \frac{dm^*}{m_t} = \int \frac{dm}{m_t} + B_{AA}M_A^2 \int \frac{m_{A,t}}{m_t} \times dm_{A,t} + B_{BB}M_B^2 \int \frac{m_{B,t}}{m_t} dm_{B,t} + \frac{B_{AB}M_AM_B}{2} \int \frac{m_{B,t}}{m_t} dm_{A,t} + \frac{B_{AB}M_AM_B}{2} \int \frac{m_{A,t}}{m_t} dm_{B,t} = \int \frac{dm}{m_t} + \frac{m_{A,t}}{(1 + \beta)} B_{AA}M_A^2 + \frac{\beta m_{B,t}}{(1 + \beta)} B_{BB}M_B^2 + \frac{\beta m_{A,t}}{2(1 + \beta)} B_{AB}M_AM_B + \frac{m_{B,t}}{2(1 + \beta)} B_{AB}M_AM_B \quad \text{(A-19)}$$

Equation A-17 now becomes

$$\ln [A^*] + \beta \ln [B^*] = (1 + \beta) \int \frac{dm}{m_t} + B_{AA}M_A^2m_{A,t} + B_{BB}M_B^2\beta m_{B,t} + \frac{B_{AB}M_AM_B}{2} \beta m_{A,t} + \frac{B_{AB}M_AM_B}{2} m_{B,t} + D \quad \text{(A-20)}$$

Subtracting $(1 + \beta) \ln m_t$ from both sides and noting that

$$(1 + \beta) \ln m_t = (1 + \beta) \int \frac{dm_t}{m_t} \quad \text{(A-21)}$$

we have

$$\ln X_A^* + \beta \ln X_B^* = (1 + \beta) \left\{ \int \frac{dm}{m_t} - \int \frac{dm_t}{m_t} \right\} + B_{AA}M_A^2m_{A,t} + B_{BB}M_B^2\beta m_{B,t} + (^1/_2)B_{AB}M_AM_BBm_{A,t} + (^1/_2)B_{AB}M_AM_Bm_{B,t} + D \quad \text{(A-22)}$$

Integrating by parts

$$\ln X_A^* + \beta \ln X_B^* = (1 + \beta)(\alpha_n^{*-1} - 1) + (1 + \beta) \int_0^{m_t} (\alpha_n^{*-1} - 1) \frac{dm_t}{m_t} + D = (1 + \beta) \times (\alpha_n^{-1} - 1) + (1 + \beta) \int_0^{m_t} (\alpha_n^{-1} - 1) \frac{dm_t}{m_t} + B_{AA}M_A^2m_{A,t} + B_{BB}M_B^2\beta m_{B,t} + (^1/_2)B_{AB}M_AM_B\beta m_{A,t} + (^1/_2)B_{AB}M_AM_Bm_{B,t} + D \quad \text{(A-23)}$$

where X_A^*, X_B^* are equal to $[A^*]/m_t$ and $[B^*]/m_t$, respectively.

The constant D may be evaluated from the limiting form of eq A-23 which is approached at very low concentrations, where α_n^{-1} approaches unity. The terms in B_{AA}, B_{BB}, and B_{AB} approach zero, X_A^* approaches $1/(1 + \beta)$, and X_B^* approaches $\beta/(1 + \beta)$. The value of D is therefore given by

$$D = \ln \{1/(1 + \beta)\} + \beta \ln \{\beta/(1 + \beta)\} \quad \text{(A-24)}$$

For the ideal case, when B_{AA}, B_{BB}, and B_{AB} are equal to zero, we have

$$\ln X_A + \beta \ln X_b = (1 + \beta)(\alpha_n^{-1} - 1) + (1 + \beta) \int_0^{m_t} (\alpha_n^{-1} - 1) \frac{dm_t}{m_t} + \ln \{1/(1 + \beta)\} + \beta \ln \{\beta/(1 + \beta)\} \quad \text{(A-25)}$$

Finally, from eq A-23 and A-25

$$\begin{aligned}\ln X_A^* + \beta \ln X_B^* &= \ln X_A + \beta \ln X_B + B_{AA}M_A{}^2m_{A,t} + B_{BB}M_B{}^2\beta m_{B,t} + (1/2)B_{AB}M_AM_B\beta m_{A,t} + \\ &\quad (1/2)B_{AB}M_AM_Bm_{B,t} = \ln \left\{X_A \exp\left(B_{AA}M_A{}^2m_{A,t} + \frac{B_{AB}M_AM_B}{2}m_{B,t}\right)\right\} + \beta \ln \left\{X_B \exp\left(B_{BB}M_B{}^2m_{B,t} + \frac{B_{AB}M_AM_B}{2}m_{A,t}\right)\right\} \qquad \text{(A-26)}\end{aligned}$$

Returning to eq A-17 and subtracting $\ln m^*$ ($= \int \mathrm{d}m^*/m^*$) from both sides, we have

$$\ln Z_A^* + \beta \ln Z_B^* = (1 + \beta)\int (\alpha_n{}^{*-1} - 1)\frac{\mathrm{d}m^*}{m^*} + \ln\{1/(1 + \beta)\} + \beta \ln \{\beta/(1 + \beta)\} = \Phi^* \qquad \text{(A-27)}$$

where Z_A^*, Z_B^* are the apparent mole fractions of A and B, respectively, and are given by

$$\begin{aligned}Z_A^* &= [A^*]/m^* \\ Z_B^* &= [B^*]/m^*\end{aligned} \qquad \text{(A-28)}$$

The quantities Z_A^* and Z_B^* are related to X_A^* and X_B^* by

$$\begin{aligned}Z_A^* &= X_A^*\frac{m_t}{m^*} \\ Z_B^* &= X_B^*\frac{m_t}{m^*}\end{aligned} \qquad \text{(A-29)}$$

If Φ^* is evaluated as a function of m^* for a series of values of β, then, by suitable interpolation, Φ^* may be obtained as a function of β for a series of (constant) values of m^*.

When m^* is constant, we have

$$m^*([A^*], [B^*]) = \text{constant} \qquad \text{(A-30)}$$

and

$$\frac{\mathrm{d}[A^*]}{\mathrm{d}[B^*]} = -\frac{\partial m^*}{\partial [B^*]}\Big/\frac{\partial m^*}{\partial [A^*]} \qquad \text{(A-31)}$$

$$\begin{aligned}\frac{\mathrm{d}\ln [A^*]}{\mathrm{d}\ln [B^*]} &= -\frac{\partial m^*}{\partial \ln [B^*]}\Big/\frac{\partial m^*}{\partial \ln [A^*]} \\ &= \frac{\mathrm{d}\ln Z_A^*}{\mathrm{d}\ln Z_B^*} = -\beta\end{aligned} \qquad \text{(A-32)}$$

Then

$$\begin{aligned}\ln Z_A^* &= -\int \beta d \ln Z_B^* \\ &= -\beta \ln Z_B^* + \int \ln Z_B^* \mathrm{d}\beta\end{aligned} \qquad \text{(A-33)}$$

Introducing the above into eq A-27 we obtain

$$\int \ln Z_B^* \mathrm{d}\beta = \Phi^* \qquad \text{(A-34)}$$

and

$$\ln Z_B^* = \left(\frac{\mathrm{d}\Phi^*}{\mathrm{d}\beta}\right)_{m^*}$$

The value of $\ln Z_B^*$ for particular values of m^* and β is thus equal to the slope of the tangent of the curve of Φ^* as a function of β for this (constant) value of m^*. Methods of numerical analysis suitable for computing $\ln Z_B^*$ have been discussed in the preceding paper (Steiner, 1968). The quantity Z_B^* may be converted into X_B^* by eq A-29. Insertion of X_B^* in eq A-23 yields X_A^*.

We turn now to the problem of correcting for nonideality and evaluating the actual dissociation constants. The second virial coefficients for the individual A and B species, B_{AA} and B_{BB}, may be obtained directly from measurements upon pure A and pure B.

If these do not self-associate, then the second virial coefficients may be obtained directly from the slopes of $\pi V_m/cRT$ as a function of c.

$$\frac{\pi V_m}{c_{A,t}RT} = \frac{1}{M_A} + B_{AA}c_{A,t}/2 \qquad \text{(A-35)}$$

where $c_{A,t}$ is the total concentration of A.

We shall postpone to a later section the discussion of methods for obtaining B_{AA} or B_{BB} when these species undergo self-association.

If B_{AA} and B_{BB} are known, the corresponding terms $(1/2)B_{AA}c_{A,t}{}^2$ and $(1/2)B_{BB}c_{B,t}{}^2$ may be subtracted from m^* yielding

$$\begin{aligned}m^{**} &\equiv m^* - (1/2)B_{AA}c_{A,t}{}^2 - (1/2)B_{BB}c_{B,t}{}^2 \\ &= m + (1/2)B_{AB}c_{A,t}c_{B,t} = m + (1/2)B_{AB}M_AM_Bm_{A,t}m_{B,t}\end{aligned} \qquad \text{(A-36)}$$

Unfortunately, B_{AB} cannot be evaluated independently. Equation A-26 is now replaced by

$$\begin{aligned}\ln X_A^{**} + \beta \ln X_B^{**} &= \ln \{X_A \exp(B_{AB}M_AM_Bm_{B,t}/2)\} + \beta \ln \{X_B \exp(B_{AB}M_AM_Bm_{A,t}/2)\} = (1 + \beta) \times \\ &\quad \left\{(\alpha^{**-1} - 1) + \int (\alpha^{**-1} - 1)\frac{\mathrm{d}m_t}{m_t}\right\} + \ln \{1/(1 + \beta)\} + \beta \ln \{\beta/(1 + \beta)\} \qquad \text{(A-37)}\end{aligned}$$

where $\alpha_n^{**} = m_t/m^{**}$

A quantity Φ^{**} may be defined by

$$\Phi^{**} = (1 + \beta)\int_0^{m^{**}} (\alpha^{**-1} - 1)\frac{\mathrm{d}m^{**}}{m^{**}} + \ln \{1/(1 + \beta)\} + \beta \ln \{\beta/(1 + \beta)\} = \ln Z_A^{**} + \beta \ln Z_B^{**} \qquad \text{(A-38)}$$

We also have

$$\ln Z_B^{**} = \left(\frac{\mathrm{d}\Phi^{**}}{\mathrm{d}\beta}\right)_{m^{**}} \qquad \text{(A-39)}$$

In analogy to eq A-29, Z_B^{**} is related to X_B^{**} by

$$Z_B^{**} = X_B^{**}\frac{m_t}{m^{**}} \qquad \text{(A-40)}$$

By the use of eq A-37 to A-40, X_A^{**} and X_B^{**} may be computed. From eq A-37, we may write

$$\begin{aligned} X_A^{**} &= X_A \exp(B_{AB}M_AM_Bm_{B,t}/2) \\ X_B^{**} &= X_B \exp(B_{AB}M_AM_Bm_{A,t}/2) \end{aligned} \qquad \text{(A-41)}$$

(From eq A-37, $(X_A^{**}/X_A)\exp(-{}^1/_2B_{AB}M_AM_Bm_{B,t}) = \{(X_B/X_B^{**})\exp(+{}^1/_2B_{AB}M_AM_Bm_{A,t})\}^{\beta}$. Since X_A^{**} is a function only of X_A and $m_{B,t}$, both sides must be equal to a constant. When $m_{B,t} = 0$, $X_A^{**} = X_A$, and the left-hand side equals unity, the value of the constant. Alternatively we also have, from eq A-37, $(X_A^{**}/X_A)\exp(-{}^1/_2B_{AB}M_A \cdot M_Bm_{B,t}) = (X_B/X_B^{**})^{\beta}\exp({}^1/_2B_{AB}M_AM_Bm_{B,t})$. Since $X_A^{**}/X_A > 1$ and $X_B/X_B^{**} < 1$, if β is varied at constant $m_{B,t}$, this can hold only if both sides equal unity.)

Also

$$\begin{aligned} [A^{**}] &= [A]\exp(B_{AB}M_AM_Bm_{B,t}/2) \\ [B^{**}] &= [B]\exp(B_{AB}M_AM_Bm_{A,t}/2) \end{aligned} \qquad \text{(A-42)}$$

If m^{**} is defined by eq A-36, then direct substitution verifies that eq A-42 represents a solution of

$$[A^{**}]\frac{\partial m^{**}}{\partial[A^{**}]} + [B^{**}]\frac{\partial m^{**}}{\partial[B^{**}]} = m_t \qquad \text{(A-43)}$$

The methods described in the preceding sections require the independent evaluation of B_{AA} and B_{BB} from measurements upon pure A and pure B. If self-association is absent, there is no difficulty and B_{AA} or B_{BB} may be computed from eq A-35. If self-association is present, the equations developed by Adams (1965a,b) are applicable for several specific cases.

If only monomer, dimer, and trimer are present, then the equation derived by Adams may be written as

$$\begin{aligned} \frac{3m_{A,t}M_A^2}{M_n^*} - M_Am_{A,t} = {} & 2M_A[A^*]\exp(-B_{AA}M_A^2m_{A,t}) + \\ & ({}^1/_2)(L + B_{AA}M_A)M_A^2[A^*]^2\exp(-2B_{AA}M_A^2m_{A,t}) + \\ & ({}^3/_2)B_{AA}M_A^3m_{A,t}^2 \end{aligned} \qquad \text{(A-44)}$$

where

$$-\frac{L}{2} \equiv \lim_{c\to 0}\frac{\mathrm{d}\alpha^{*-1}}{\mathrm{d}c_{A,t}};\ \alpha^* = \frac{M_n^*}{M_A} \qquad \text{(A-45)}$$

B_{AA} is obtained by numerical solution of A-44.

In the more general case, where no upper limit can be set to the degree of association, the computational methods developed by Van Holde *et al.* (1969) may be useful.

An alternative approach may be developed which depends upon the expansion of m^* or m_t in a power series in terms of experimentally obtainable quantities. If $m_{A,t}$ is expressed as a series expansion in $[A^*] = [A]\exp(B_{AA}M_A^2m_{A,t})$, then

$$\begin{aligned} m_{A,t} = {} & \left(\frac{\mathrm{d}m_{A,t}}{\mathrm{d}[A^*]}\right)_0[A^*] + {}^1/_2\left(\frac{\mathrm{d}^2m_{A,t}}{\mathrm{d}[A^*]^2}\right)_0[A^*]^2 + \\ & {}^1/_6\left(\frac{\mathrm{d}^3m_{A,t}}{\mathrm{d}[A^*]^2}\right)_0[A^*]^3 + \ldots \end{aligned} \qquad \text{(A-46)}$$

and

$$\frac{\mathrm{d}}{\mathrm{d}[A^*]} = \frac{\mathrm{d}[A]}{\mathrm{d}[A^*]}\frac{\mathrm{d}}{\mathrm{d}[A]} \qquad \text{(A-47)}$$

where

$$\begin{aligned} \frac{\mathrm{d}[A]}{\mathrm{d}[A^*]} = {} & \exp(-B_{AA}M_A^2m_{A,t}/\{1 + B_{AA}M_A)^2\Sigma i^2K_{i0}[A^*] \times \\ & \exp(-B_{AA}M_A^2m_{A,t})^i\} \end{aligned} \qquad \text{(A-48)}$$

Using eq A-46 and A-47, the expansion takes the form

$$m_t = [A^*] + 2\sigma_{20}[A^*]^2 + 3\sigma_{30}[A^*]^3 + \ldots \qquad \text{(A-49)}$$

where

$$\begin{aligned} \sigma_{20} &= K_{20} - ({}^1/_2)B_{AA}M_A^2 \\ \sigma_{30} &= K_{30} - B_{AA}M_A^2(2K_{20} - ({}^1/_2)B_{AA}M_A^2) \\ &= K_{30} - B_{AA}M_A^2(K_{20} + \sigma_{20}) \\ &= K_{30} - B_{AA}M_A^2(2\sigma_{20} + ({}^1/_2)B_{AA}M_A^2) \end{aligned}$$

If the system is treated as if it were ideal and $[A^*]$ assumed to be equal to [A], the quantities σ_{20} and σ_{30} are obtained in place of K_{20} and K_{30}, respectively.

Since

$$\begin{aligned} [A] &= [A^*]\exp(-B_{AA}M_A^2m_{A,t}) \\ &= [A^*]\exp(2\sigma_{20}m_{A,t} - 2K_{20}m_{A,t}) \end{aligned} \qquad \text{(A-50)}$$

we may define

$$[A]\exp(2K_{20}m_{A,t}) = [A^*]\exp(2\sigma_{20}m_{A,t} \equiv \gamma) \qquad \text{(A-51)}$$

The quantity γ does not involve B_{AA} explicitly and may be defined in terms of measurable quantities. The quantity $m_{A,t}$ may be expanded in terms of γ

$$\begin{aligned} m_{A,t} = {} & \left(\frac{\mathrm{d}m_{At}}{\mathrm{d}\gamma}\right)_0\gamma + {}^1/_2\left(\frac{\mathrm{d}^2m_{A,t}}{\mathrm{d}\gamma^2}\right)_0\gamma^2 + \\ & {}^1/_6\left(\frac{\mathrm{d}^3m_{A,t}}{\mathrm{d}\gamma^3}\right)_0\gamma^3 + \ldots \end{aligned} \qquad \text{(A-52)}$$

and

$$\frac{d}{d\gamma} = \frac{e^{-2K_{20}m_{A,t}}}{1 + 2K_{20}\Sigma i^2 K_{i0}[A]^i}\frac{d}{d[A]} \qquad \text{(A-53)}$$

The expansion of $m_{A,t}$ in terms of γ, using eq A-52 and A-53 assumes the form

$$m_{A,t} = \gamma + 3(K_{30} - 2K_{20}^2)\gamma^3 + \ldots \qquad \text{(A-54)}$$

The coefficient of γ^2 is zero in this expansion. Upon taking the limit when γ^2 and [A] equal zero

$$\lim_{\gamma \to 0}\left[\frac{(m_{A,t}/\gamma) - 1}{3\gamma^2}\right] = K_{30} - 2K_{20}^2 \equiv \sigma'_{30} \qquad \text{(A-55)}$$

This provides a relationship between K_{30} and K_{20}. From eq A-49 and A-55, we have

$$\sigma_{30} = \sigma'_{30} + 2(\sigma_{20} + (1/2)B_{AA}M_A^2)^2 - B_{AA}M_A^2(2\sigma_{20} + (1/2)B_{AA}M_A^2) \qquad \text{(A-56)}$$

If σ_{30}, σ_{20}, and σ'_{30} are known with sufficient accuracy, eq A-56 may be solved for B_{AA}, which is the only unknown. Equation A-56 provides, in principle, a general method for obtaining B_{AA} which does not require any assumptions as to model. In practice data of very high accuracy would be required to obtain σ_{30}, σ_{20}, and σ'_{30} with the necessary accuracy.

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Observations on the Conformation of Human Serum High-Density Lipoproteins Using Infrared Spectroscopy, Circular Dichroism, and Electron Spin Resonance*

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ABSTRACT: Human serum high-density lipoproteins (HDL) were examined before and after delipidation by the techniques of infrared spectroscopy, by circular dichroism, and by electron spin resonance. Comparisons were made with the techniques under identical conditions of the buffer system, protein concentration, and temperature. In D_2O solution the infrared band associated with the amide I resonance of HDL and apoHDL had its major absorption at 1637–1640 cm^{-1} with shoulders at about 1630 and 1650 cm^{-1}. Circular dichroic spectra were characteristic of a high helical content as previously described by others. The electron spin resonance spectrum of HDL, after spin labeling with *N*-(1-oxy-2,2,6,6-tetramethylpiperidinyl)maleimide contained signals associated with both weakly (narrow signal) and strongly (broad signal) constrained spin label. The strongly constrained signal was decreased in relative intensity at high temperatures, was increased in relative intensity at low temperatures and was practically abolished by delipidation. The change caused in the electron spin resonance spectrum by delipidation was qualitatively much greater than any detected by infrared or circular dichroism spectroscopy. It was concluded that the changes in the electron spin resonance spectrum consequent upon delipidation were more likely related to change in the local environment of the spin label rather than to large changes in the secondary structure of the molecule. The helical conformation, much of which was to be retained by apoHDL, appeared to exhibit its major infrared band associated with the amide I vibration at a significantly lower frequency than does the helical conformation in other proteins such as myoglobin.

The conformational and structural properties of human serum high-density lipoproteins (HDL)[1] have been investigated by a number of techniques, including optical rotatory dispersion (Scanu, 1965), circular dichroism (Scanu and Hirz, 1968), nuclear magnetic resonance (Steim *et al.*, 1968; Chapman *et al.*, 1969a,b), and recently by electron spin resonance (Gotto and Kon, 1969; Gotto *et al.*, 1970). The

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[1] The following abbreviations are used: HDL, human serum high-density lipoproteins of d = 1.063–1.21; apoHDL, the protein moiety of HDL after delipidation with ether–ethanol; *N*-(1-oxy-2,2,6,6-tetramethylpiperidinyl)maleimide, nitroxide radical I.