Sedimentation Equilibrium in Reacting Systems. III. Evaluation of the Number Average ($M_{n(c)}$) Molecular Weight, Equilibrium Constants, and Nonideal Effects*

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ABSTRACT: In aqueous solutions many proteins and detergents undergo associations of the type $n\,P_1 \rightleftarrows mP_2 + qP_3 + \ldots$ or $nP_1 \rightleftarrows P_n$, $n = 2, 3, \ldots$. When the logarithm of the activity coefficient for each associating species is defined by $\ln y_i = iBM_1c + O(c^2)$, with $i = 1, 2, \ldots$, then the total concentration for ideal or nonideal solutions can be represented as $c = c_1 + K_2c_1^2 + K_3c_1^3$ or $c = c_1 + K_nc_1^n$. From the definition of the number average molecular ($M_{n(c)}$) weight one has $cM_1/M_{n(c)} = M_1 \sum_{i=1}^{n} c_i/M_i$; it is shown that this may also be obtained from the relation $cM_1/M_{n(c)} = \int_0^c M_1/M_{w(c)}dc$, where $M_{w(c)}$ is the weight average molecular weight. For nonideal solutions one obtains $cM_1/M_{n\,app} = \int_0^c M_1/M_{w\,app}\,dc$. These relations, along with other previously derived quantities, can be used to analyze ideal or nonideal associations; in addition they can be combined with $M_{w(c)}$ or $M_{w\,app}$ for this purpose. Other conventions for representing the activity coefficient of associating species are discussed. It is also shown how to formulate the sedimentation equilibrium equations for ionizable, associating systems.

Many proteins (Reithel, 1963) and detergents (Debye, 1947, 1949; Ludlum, 1956; Anacker *et al.*, 1964) undergo associations of the type

$$nP_1 \rightleftarrows qP_2 + mP_3 + \ldots \quad (1)$$

or

$$nP_1 \rightleftarrows P_n,\ n = 2, 3, \ldots \quad (2)$$

in solution. In a previous publication Adams and Williams (1964) developed a method for testing the ideality of associating systems described by equations (1) or (2). They were able to develop methods for analyzing the nonideal monomer-dimer or monomer-*n*-mer ($n \geq 3$) systems; however, no satisfactory treatment was obtained for the nonideal monomer-dimer-trimer equilibrium. Their methods were based on the weight average (M_{wr}) molecular weight for ideal solutions, or the apparent weight ($M_{wr\,app}$) average molecular weight for nonideal solutions, the subscript r indicating that this is the value at any radial position in the ultracentrifuge cell between the meniscus and the bottom of the solution column.

The purpose of this communication is to show how the number average (M_{nr}) molecular weight can be obtained for ideal associating systems described by equations (1) and (2). For nonideal systems it will be shown how one can develop an apparent number average ($M_{nr\,app}$) molecular weight. Furthermore, it will be shown how $M_{n\,app}$,[1] $M_{w\,app}$,[1] or both combined can be used to evaluate equilibrium constants and nonideality terms. This treatment will include the monomer-dimer-trimer system as well as other associating systems described by equations (1) and (2). These quantities are evaluated at *real* concentrations instead of infinite dilution of the macromolecular component. In addition to a consideration of two component, neutral systems, it will be shown how the Casassa-Eisenberg (1960, 1964; Eisenberg and Casassa, 1960) definition of components can be applied to the development of sedimentation equilibrium equations for three or more component systems containing charged, associating macromolecules.

Evaluation of M_{nr} and $M_{n\,app}$

For two component, neutral associating systems of the types given by equations (1) and (2), the condition of chemical equilibrium is

$$n\mu_1 = \mu_n,\ n = 2, 3, \ldots \quad (3)$$

where μ_1 is the chemical potential per mole of monomer

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[1] For convenience the quantities $M_{wr\,app}$ and $M_{nr\,app}$ will be written as $M_{w\,app}$ and $M_{n\,app}$, respectively.

and μ_n is the chemical potential/mole of species n. Following the previous convention (Adams and Fujita, 1963; Adams and Williams, 1964), the activity coefficient for each associating species i is defined by the equation

$$\ln y_i = iBM_1c + 0(c^2),\ i = 1, 2, \ldots \quad (4)$$

For ideal systems $BM_1 = 0$. As a result of equations (3) and (4), the total concentration for ideal or nonideal systems can be expressed as

$$c = c_1 + K_2c_1^2 + K_3c_1^3 \quad (5)$$

for a monomer-dimer-trimer equilibrium, or

$$c = c_1 + K_nc_1^n,\ n = 2, 3 \ldots \quad (5a)$$

for a monomer-n-mer equilibrium. The equilibrium constants are defined by

$$K_n = a_n/a_1^n = c_n/c_1^n,\ n = 2, 3 \ldots \quad (5b)$$

since

$$y_n/y_1^n = 1 \quad (5c)$$

as a consequence of equation (4). Equations will now be developed that allow M_{nr} or $M_{nr\ \mathrm{app}}$ to be evaluated from a series of sedimentation equilibrium experiments at different initial concentrations.

The Monomer-Dimer-Trimer Equilibrium. The total concentration for the associating solute in a monomer-dimer-trimer equilibrium is given by equation (5). In sedimentation equilibrium experiments $c = c_r$, the concentration at any radial position in the solution column between the meniscus ($r = a$) and the cell bottom ($r = b$). The number average ($M_{n(c)}$) molecular weight is defined as

$$M_{n(c)}\ (\equiv M_{nr}) = \sum_i n_iM_i/\sum_i n_i = c/\sum_i (c_i/M_i) \quad (6)$$

where c_i is the concentration of associating species i, n_i is the number of moles of species i, M_i is the molecular weight of species i, and c is the total concentration. It should be noted that the quantities c_i, c, and n_i refer to their values at any radial position r, i.e., $c_i = c_{ir}$, etc., between the meniscus position ($r = a$) and the position of the cell bottom ($r = b$). For convenience the subscript r will be omitted when writing c, c_i, or n_i, except in the brief discussion that follows.

Since we are concerned with chemically reacting systems, M_n depends on the total concentration ($c = c_r$), i.e., $M_n = f(c)$; this is also true with osmotic pressure measurements on these systems! Thus it is convenient to use the symbol $M_{n(c)}$ to indicate that one is dealing with associating systems. The relation between the M_{nr} obtained from sedimentation equilibrium experiments and the M_n obtained from osmotic pressure experiments can be readily shown. If osmotic pressure experiments, as well as sedimentation equilibrium experiments, were performed on the same associating system, a monomer-dimer-trimer system for example, then whenever c(osmometer) = c_r(sedimentation equilibrium) one has $c_{1(\mathrm{osm})} + K_2c_{1(\mathrm{osm})}^2 + K_3c_{1(\mathrm{osm})}^3 = c_{1r(\mathrm{sed\ equil})} + K_2c_{1r(\mathrm{sed\ equil})}^2 + K_3c_{1(\mathrm{sed\ equil})}^3$. Since K_2 and K_3 are constants, one finds that $c_{1(\mathrm{osm})} = c_{1r(\mathrm{sed\ equil})}$ and also that $M_{nr(\mathrm{sed\ equil})} = M_{n(\mathrm{osm})} = M_{n(c)}$ *whenever* $c_{(osm)} = c_{r(sed\ equil)}$. The quantity $M_{n\ \mathrm{cell}}$ defined by Lansing and Kraemer (1935) is *not* needed in sedimentation equilibrium experiments on associating systems described by equations (1) and (2), and $M_{n\ \mathrm{cell}}$ is *not* the number average molecular weight of the original solution, i.e., $M_{n\ \mathrm{cell}} \neq M_n$ at $c = c_0$. This can be proven using arguments analogous to those used in showing $M_{w\ \mathrm{cell}} \neq M_w$ at $c = c_0$ (Adams, 1964) in associating systems described by equations (1) and (2).

Equation (6) can be written as

$$c/M_{n(c)} = \sum_i c_i/M_i \quad (6a)$$

For the association equilibrium under discussion, equation (6a) becomes

$$c/M_{n(c)} = \frac{c_1}{M_1} + \frac{K_2c_1^2}{2M_1} + \frac{K_3c_1^3}{3M_1} \quad (6b)$$

More conveniently, we can write equation (6b) as

$$\frac{cM_1}{M_{n(c)}} = c_1 + \frac{K_2c_1^2}{2} + \frac{K_3c_1^3}{3} \quad (6c)$$

Equation (6c) can also be obtained from the following integral

$$\int_0^{c_1} \frac{c}{c_1}\,dc_1 = \int_0^{c_1} (1 + K_2c_1 + K_3c_1^2)dc_1$$

$$= c_1 + \frac{K_2c_1^2}{2} + \frac{K_3c_1^3}{3} \quad (7)$$

From equation (5) it can be shown that

$$dc_1 = dc/(1 + 2K_2c_1 + 3K_3c_1^2) \quad (7a)$$

Thus,

$$\int_0^{c_1} \frac{c}{c_1}\,dc_1 = \int_0^{c_1} \frac{1 + K_2c_1 + K_3c_1^2}{1 + 2K_2c_1 + 3K_3c_1^2}\,dc \quad (7b)$$

However, Adams and Williams (1964) and also Adams and Fujita (1963) have shown

$$\frac{M_1}{M_{w(c)}} = \frac{1 + K_2c_1 + K_3c_1^2}{1 + 2K_2c_1 + 3K_3c_1^2} \quad (7c)$$

for this type of association. Here $M_{w(c)}$ is the weight

average molecular weight at any radial position, i.e., $M_{w(c)} = M_{wr}$. Therefore, it follows from equations (6c, 7a, 7b) and (7c) that

$$\int_0^c \frac{M_1}{M_{w(c)}}\,dc = cM_1/M_{n(c)} \tag{8}$$

or

$$\int_0^c \frac{dc}{M_{w(c)}} = c/M_{n(c)} \tag{8a}$$

Since we are dealing with associating systems we write $M_{n(c)}$ to indicate that M_n varies with c (as do the other average molecular weights). It should be noted that $M_{n(c)} = M_{nr}$, the number average molecular weight at any radial position in the ultracentrifuge cell.

For nonideal solutions we do not know $M_{w(c)}$, instead we measure $M_{w\ \mathrm{app}}$. In this case one obtains

$$\int_0^c \frac{M_1}{M_{w\ \mathrm{app}}}\,dc = \int_0^c \frac{M_1}{M_{w(c)}}\,dc + \int_0^c BM_1c\,dc = \frac{cM_1}{M_{n(c)}} + \frac{BM_1c^2}{2} = \frac{cM_1}{M_{n\ \mathrm{app}}} \tag{9}$$

It has been shown previously (Adams and Fujita, 1963; Adams and Williams, 1964) that

$$\frac{M_1}{M_{w\ \mathrm{app}}} = \frac{M_1}{M_{w(c)}} + BM_1c \tag{9a}$$

Although equation (9) defines $M_{n\ \mathrm{app}}$ for the monomer-dimer-trimer association, the treatment can be extended to the more general case of monomer-dimer-n-mers, as can equations (8) and (8a). It should be emphasized that these derivations for $M_{n(c)}$ and $M_{n\ \mathrm{app}}$ are only valid for associating systems described by equations (1) or (2).

In order to evaluate $M_{n(c)}$ or $M_{n\ \mathrm{app}}$ from equations (8) or (9), it is necessary to perform a series of sedimentation equilibrium experiments at different initial concentrations. The values of $M_1/M_{w(c)}$ (or $M_1/M_{w\ \mathrm{app}}$) so obtained are plotted against c; one recalls for associating systems described by equations (1) and (2) that the $\lim_{c\to 0} M_1/M_{w(c)} = 1$ and the $\lim_{c\to 0} M_1/M_{w\ \mathrm{app}} = 1$. The area under the curve (of $M_1/M_{w(c)}$ versus c or $M_1/M_{w\ \mathrm{app}}$ versus c) between $c = 0$ and $c = c$ gives $cM_1/M_{n(c)}$ or $cM_1/M_{n\ \mathrm{app}}$; the required integration can be done using Simpson's rule or the trapezoidal rule. Incidentally one notes for associating systems of the types described by equations (1) and (2) that the $\lim_{c\to 0} M_1/M_{n(c)} = 1$ and the $\lim_{c\to 0} M_1/M_{n\ \mathrm{app}} = 1$. In general one cannot obtain $M_{n(c)}$ from a single experiment. As Lansing and Kraemer (1935) point out, one obtains an equation in two unknowns, since $(c/M_n)_r - (c/M_n)_a = A\int_a^r cd(r^2)$, $A = (1 - \bar{v}\rho)\omega^2/2RT$, for ideal solutions, unless one uses extremely high speeds (Wales *et al.*, 1951; Yphantis, 1964) where c_a is essentially zero. The high speeds limit the experiments to very dilute solutions, since at higher concentrations one gets defocusing in the vicinity of the cell bottom; in addition with high speed experiments one may get pressure effects, salt redistribution effects, possible gel formation, and optical distortion. These disadvantages are overcome by performing the series of sedimentation equilibrium experiments at lower speeds, and then plotting the values of $M_1/M_{w(c)}$ (or $M_1/M_{w\ \mathrm{app}}$) versus c to obtain $cM_1/M_{n(c)}$ (or $cM_1/M_{n\ \mathrm{app}}$) by equations (8) or (9). Although this procedure is more tedious, it may be more accurate and it certainly does permit the evaluation of $M_{n(c)}$ or $M_{n\ \mathrm{app}}$ over a much larger concentration range.

The Monomer-Dimer and Monomer-n-mer Equilibria. With either the monomer-dimer or the monomer-n-mer ($n \geq 3$) equilibrium one can obtain $M_{n(c)}$ in the same manner. The total concentration for these associations is given by equation (5a). Thus

$$\frac{cM_1}{M_{n(c)}} = c_1 + \frac{K_nc_1^n}{n} = \int_0^{c_1} \frac{c}{c_1}\,dc_1 = \int_0^c \frac{1 + K_nc_1^{(n-1)}}{1 + nK_nc_1^{(n-1)}}\,dc = \int_0^c \frac{M_1}{M_{w(c)}}\,dc \tag{10}$$

where $n = 2, 3, \ldots$. In nonideal solutions one obtains

$$\int_0^c \frac{M_1}{M_{w\ \mathrm{app}}}dc = \frac{cM_1}{M_{n(c)}} + \frac{BM_1c^2}{2} = \frac{cM_1}{M_{n\ \mathrm{app}}} \tag{10a}$$

since for these cases

$$\frac{M_1}{M_{w\ \mathrm{app}}} = \frac{M_1}{M_{w(c)}} + BM_1c = \frac{1 + K_nc_1^{(n-1)}}{1 + nK_nc_1^{(n-1)}} + BM_1c \tag{10b}$$

After performing a series of sedimentation equilibrium experiments at various initial concentrations, $M_1/M_{w(c)}$ or $M_1/M_{w\ \mathrm{app}}$ are plotted against c. Equations (10) or (10a) are evaluated numerically [in the same manner as were equations (8) or (9)] from these plots, using Simpson's rule or the trapezoidal rule.

Evaluation of the Equilibrium Constants and the Nonideality Term

There are several ways one could evaluate equilibrium constants and the nonideality term. Method 1 uses $M_{n\ \mathrm{app}}$; method 2 uses $M_{w\ \mathrm{app}}$; and method 3 uses both $M_{n\ \mathrm{app}}$ and $M_{w\ \mathrm{app}}$. These methods will be developed in detail for the monomer-dimer-trimer equilibrium; they will then be extended to the monomer-n-mer equilibrium.

The Monomer-Dimer-Trimer Equilibrium. METHOD 1. Using equations (5), (6c), and (9), we find

$$\frac{3cM_1}{M_{n\ \mathrm{app}}} - c = 2c_1 + \frac{K_2c_1^2}{2} + \frac{3BM_1c^2}{2} \qquad (11)$$

At this point quantities which have been derived previously (Adams and Williams, 1964) will be introduced; these quantities can be evaluated from the experimental data.

$$\alpha = c_1e^{BM_1c} \text{ or } c_1 = \alpha e^{-BM_1c} \qquad (11a)$$

$$L = K_2 - BM_1 \text{ or } K_2 = L + BM_1 \qquad (11b)$$

$$\beta = c_1e^{K_2c} \text{ or } c_1 = \beta e^{-K_2c} \qquad (11c)$$

When equations (11a) and (11b) are substituted into equation (11), we obtain

$$\frac{3cM_1}{M_{n\ \mathrm{app}}} = c + 2\alpha e^{-BM_1c} + \frac{(L + BM_1)\alpha^2e^{-2BM_1c}}{2} + \frac{3BM_1c^2}{2} \qquad (11d)$$

Equation (11d) has only one unknown, the quantity BM_1; at any chosen c and $M_{n\ \mathrm{app}}$, it may be solved by successive approximations. Since K_2 may be greater than BM_1, we may use equations (11b) and (11c) together with equation (11) to obtain

$$\frac{3cM_1}{M_{n\ \mathrm{app}}} = c + 2\beta e^{-K_2c} + \frac{K_2\beta^2}{2}e^{-2K_2c} + \frac{3(K_2 - L)}{2}c^2 \qquad (11e)$$

which has only K_2 as an unknown. This equation may be more sensitive than equation (11d), although it may be unmanageable for very large values of K_2. Once BM_1 or K_2 has been evaluated one can obtain c_1, c_2 (i.e., $K_2c_1^2$), and K_2 or BM_1 by comparison of the terms between equations (11) and (11d) or (11e). In addition one can obtain c_1 from equations (11a) or (11c) and K_2 or BM_1 from equation (11b). The quantity K_3 is obtained from equation (5); thus

$$K_3 = (c - c_1 - K_2c_1^2)/c_1^3 \qquad (12)$$

The quantities $M_{n(c)}$ and $M_{w(c)}$ are evaluated from equations (9) and (9a), respectively.

METHOD 2. By suitably combining equations (5), (7c), and (9a), one finds

$$\frac{M_1}{cM_{w\ \mathrm{app}}} = 1/(c_1 + 2K_2c_1^2 + 3K_3c_1^3) + BM_1 \qquad (13)$$

This may be rearranged to give

$$\frac{1}{\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} = c_1 + 2K_2c_1^2 + 3K_3c_1^3 \qquad (13a)$$

Subtracting equation (13) from the quantity (3c) and then applying equations (11a) and (11b), one obtains

$$3c = \frac{1}{\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} + 2\alpha e^{-BM_1c} + (L + BM_1)\alpha^2e^{-2BM_1c} = \frac{1}{\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} + 2c_1 + K_2c_1^2 \qquad (13b)$$

At any chosen value of c and $M_{w\ \mathrm{app}}$ one can solve equation (13b) for BM_1 by successive approximations. Alternatively one can apply equations (11b) and (11c) to obtain an equation in one unknown, K_2. The second and third terms on the right side of equation (13b) are c_1 and c_2 ($K_2c_1^2$), respectively. One can evaluate the other quantities K_2 or BM_1, etc., in the same manner as was described for method 1.

METHOD 3. On subtracting equation (11) from equation (13b) one obtains

$$4c - \frac{3cM_1}{M_{n\ \mathrm{app}}} = \frac{1}{\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} + \frac{K_2c_1^2}{2} - \frac{3BM_1c^2}{2} \qquad (14)$$

By using equations (11a) and (11b), we can rearrange equation (14) to give an equation in one unknown, BM_1; thus

$$4c - \frac{3cM_1}{M_{n\ \mathrm{app}}} = \frac{1}{\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} + \frac{(L + BM_1)\alpha^2e^{-2BM_1c}}{2} - \frac{3BM_1c^2}{2} \qquad (14a)$$

One could also use equations (11b), (11c), and (14) to give an equation analogous to equation (14a) in the unknown K_2. Either way, at any chosen values of c, $M_{w\ \mathrm{app}}$, and $M_{n\ \mathrm{app}}$ the equations are solved by successive approximations of the unknown. The quantity $K_2c_1^2$ is obtained from the second term on the right in equation (14a); the other quantities can be evaluated in the manner described at the end of method 1. One need not use the quantity L (equation 11b) to solve the nonideal monomer-dimer-trimer equilibrium; thus from equations (11, 11a) and (13b) one obtains

$$\frac{6cM_1}{M_{n\ \mathrm{app}}} - 5c = 2c_1 + 3BM_1c^2 - \frac{1}{\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} = 2\alpha_1e^{-BM_1c} + 3BM_1c^2 - \frac{1}{\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} \qquad (14b)$$

One could use a variation of Method 3 to evaluate a nonideal monomer-dimer-trimer-tetramer equilibrium; at present it appears that this is the limit of this method of analysis for the monomer-dimer-n-mers type of equilibrium, unless new relations are discovered.

The Monomer-Dimer Equilibrium. All three methods may be used in analyzing monomer-dimer equilibria. For this type of association, equation (5a) becomes $c = c_1 + K_2c_1^2$. With method 2, for example, and using equations (5a, 10b) and (11a), one obtains

$$2c = c_1 + 1 \Big/ \left(\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1 \right) \qquad (15)$$

$$2c = \alpha e^{-BM_1c} + 1 \Big/ \left(\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1 \right) \qquad (15a)$$

At any chosen value of c and $M_{w\ \mathrm{app}}$, equation (15a) may be solved by successive approximations of the unknown, BM_1. The first term on the left of equation (15a) is c_1, and one may use it and c, the total concentration, to obtain K_2, or one may obtain K_2 from equation (11b). The quantities $M_{n(c)}$ and $M_{w(c)}$ are evaluated from equations (10a) and (10b), respectively.

The Monomer-n-mer Equilibrium. The monomer-n-mer equilibrium poses some interesting problems. If n, the degree of aggregation, is known, then one can readily apply methods 1, 2, or 3. For example, method 2 will give

$$nc = (n - 1)c_1 + 1 \Big/ \left(\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1 \right) \qquad (16)$$

$$nc = (n - 1)\alpha e^{-BM_1c} + 1 \Big/ \left(\frac{M_1}{cM_{w\ \mathrm{app}}} - BM_1 \right) \qquad (16a)$$

Here we have used equations (5a), (10b), and (11a); it has been noted previously (Adams and Williams, 1964) that equation (11a) is applicable to all associating systems characterized by equations (1) and (2), provided that the activity coefficient of each associating species can be described by equation (4). Equation (16a) has one unknown, BM_1, and it can be evaluated by successive approximations of the unknown. The other desired quantities can be evaluated by the methods discussed in the previous sections.

It should be noted that a method has been developed to obtain BM_1 (Adams and Williams, 1964) in nonideal monomer-n-mer systems since

$$\lim_{c \to 0} dM_{w\ \mathrm{app}}/dc = -BM_1^2$$

or

$$\lim_{c \to 0} d(M_1/M_{w\ \mathrm{app}})/dc = BM_1$$

For positive BM_1, a plot of $M_{w\ \mathrm{app}}$ against c will have a minimum near but not at $c = 0$ (the corresponding plot of $M_1/M_{w\ \mathrm{app}}$ against c will show a maximum here); hence it may be difficult to evaluate the limiting slope to obtain BM_1. Thus it may be more advantageous to use equation (16a) at real concentrations.

If n, the degree of aggregation, is unknown, then one can use both $M_{w\ \mathrm{app}}$ and $M_{n\ \mathrm{app}}$ to evaluate n, BM_1, etc. Starting with equation (10b), one may obtain

$$n = \left[\frac{1}{\dfrac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} - c_1 \right] \Big/ K_nc_1^n \qquad (17)$$

Using equation (11a) and remembering that equation (5a) gives $K_nc_1^n = c - c_1$, one finds

$$n = \left[\frac{1}{\dfrac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} - \alpha e^{-BM_1c} \right] \Big/ (c - \alpha e^{-BM_1c}) \qquad (17a)$$

In equation (17a) the quantity n is expressed in terms of the other unknown BM_1. To reduce everything to one unknown, one first uses equation (5a) to modify equation (10a); this result is combined with equation (11a) to give another equation in n and BM_1. Thus

$$cM_1/M_{n\ \mathrm{app}} = c_1 + \frac{(c - c_1)}{n} + \frac{BM_1c^2}{2}$$

$$= \alpha e^{-BM_1c} + \frac{(c - \alpha e^{-BM_1c})}{n} + \frac{BM_1c^2}{2} \qquad (18)$$

When one substitutes equation (17a) into equation (18), one obtains

$$cM_1/M_{n\ \mathrm{app}} = \alpha e^{-BM_1c} + \frac{BM_1c^2}{2} + \frac{(c - \alpha e^{-BM_1c})^2}{\left[\dfrac{1}{\dfrac{M_1}{cM_{w\ \mathrm{app}}} - BM_1} - \alpha e^{-BM_1c} \right]} \qquad (19)$$

Thus at any chosen value of c, $M_{n\ \mathrm{app}}$, $M_{w\ \mathrm{app}}$, and α, one can solve equation (19) for BM_1 by successive approximations; the quantity c_1 can be obtained from the first term on the right in equation (19) or from equation (11a). The degree of aggregation, n, can be obtained from equations (17a) or (18); K_n is obtained from equation (5a); and the quantities $M_{n(c)}$ or $M_{w(c)}$ are available from equations (10a) and (10b), respectively.

The Use of Two Virial Coefficients

If the activity coefficient is represented by

$$\ln y_i = iB_1M_1c + iB_2M_1c^2 + 0(c^3), \qquad i = 1, 2, \ldots \qquad (20)$$

then it has been shown previously (Adams and Williams, 1964) that

$$M_1/M_{w\ \mathrm{app}} = M_1/M_{w(c)} + B_1M_1c + 2B_2M_1c^2 \quad (21)$$

In addition it was shown that

$$c = c_1 + K_2c_1^2 + K_3c_1^3 + \ldots \quad (21a)$$

for the monomer-dimer-trimer (and n-mer) association or

$$c = c_1 + K_nc_1^n,\ n = 2, 3 \ldots \quad (21b)$$

for the monomer-n-mer association, and also

$$\alpha = c_1e^{B_1M_1c + B_2M_1c^2} \quad (21c)$$

for the association equilibria described by equations (1) or (2). When equation (20) applies, we can also obtain

$$\beta = c_1e^{K_2c + B_2M_1c^2} \quad (21d)$$

Whenever equation (20) is applicable, the equation for the apparent number ($M_{n\ \mathrm{app}}$) average molecular weight becomes

$$cM_1/M_{n\ \mathrm{app}} = \int_0^c \frac{M_1}{M_{w\ \mathrm{app}}}\,dc = \frac{cM_1}{M_{n(c)}} + \frac{B_1M_1c^2}{2} + \frac{2B_2M_1c^3}{3} \quad (22)$$

The relations given in this section can be used to find a quantity σ, which is defined as

$$\sigma = B_1M_1 + B_2M_1c \quad (23)$$

$$d\sigma/dc = B_2M_1 \quad (23a)$$

Using equations (21, 21a, 21c) and (23) with the non-ideal monomer-dimer-trimer association as an example, we find

$$3c - 1\Big/\left[\frac{M_1}{cM_{w\ \mathrm{app}}} - (\sigma + B_2M_1c)\right] = 2\alpha e^{-\sigma c} + (L + \sigma - B_2M_1c)\alpha^2e^{-2\sigma c} \quad (24)$$

$$B_2M_1 = \frac{\dfrac{3cM_1}{M_{n\ \mathrm{app}}} - c - \left[2\alpha e^{-\sigma c} + (L + \sigma)\dfrac{\alpha^2e^{-\sigma c}}{2} + \dfrac{3}{2}\sigma c^2\right]}{(c^3 - \alpha^2ce^{-2\sigma c})/2} \quad (25)$$

Equation (25) can be substituted into equation (24), and the resulting equation in one unknown, σ, can be solved by successive approximations at any chosen value of α, c, $M_{w\ \mathrm{app}}$, and $M_{n\ \mathrm{app}}$. Equation (23a) shows that one can obtain B_2M_1 from the slope of a plot of σ against c. Analogous equations could be derived for the monomer-n-mer equilibria. Instead of using α, one could use β, defined by equation (21d); in this instance one would determine a new quantity γ, such that $\gamma = K_2 + B_2M_1c$.

There exists the possibility of extreme nonideality, such as discussed by Adams and Williams (1964) in their equations (44) to (51a). In this case the theory developed here would not apply.

Multicomponent, Charged Systems

Sedimentation equilibrium experiments on proteins and detergents involve aqueous solutions containing a macromolecule, which may dissociate into ions, and one or more supporting electrolytes to reduce the charge effects, act as a buffer, or do both. The problem of defining components in solutions containing ionizing macromolecules, as well as the working equations for osmotic pressure, light scattering, and sedimentation equilibrium, has received a lot of interest. This subject has been recently reviewed by Casassa and Eisenberg (1964), and references to the literature on the subject will be found in their article. Here we wish to utilize the Casassa-Eisenberg (1960, 1964; Eisenberg and Casassa, 1960) treatment to develop definitions for the associating, macroionic species, as well as working equations for the sedimentation equilibrium of these systems.

Consider a three-component system containing a solvent, water, a supporting electrolyte, BX, and an associating ionizing macromolecule, PX_z. Following standard convention (Johnson *et al.*, 1954, 1957; Casassa and Eisenberg, 1960, 1964; Eisenberg and Casassa, 1960), the components are labeled as follows:

$$1 = H_2O$$

$$2 = PX_z$$

$$3 = BX$$

Even numbers are reserved for the macromolecule, one is reserved for the solvent (water), and odd numbers other than one for the supporting (diffusible) electrolytes. For simplicity in the treatment that follows it will be assumed that the macromolecule undergoes a monomer-dimer association, although the treatment may be extended to other association equilibria described by equations (1) and (2).

When the associating macromolecule is dissolved in the supporting electrolyte solution and subsequently dialyzed, there will be an unequal distribution of ions on both sides of the dialysis membrane due to the Gibbs-Donnan effect. One convenient way of defining components for a three- (or multi-) component system is to set the supporting electrolyte concentration the same on both sides of the membrane; in doing this one includes ν_{2i} moles of diffusible ions, common to the

supporting electrolyte or electrolytes, in the formulation of component 2, the macromolecule. Thus, the activity of the monomeric species becomes

$$\ln a_{2M} = \ln (W_{2M}/M_M) + \nu_{2B} \ln (W_B/M_B) + \nu_{2X} \ln (W_z/M_X) + \gamma_2 \quad (26)$$

Here ν_{2i} is the number of moles of diffusible ionic species i (B or X) included in 1 mole of the monomeric species, and W_i is the weight of species i/kg solvent. Equation (26) is based on the conventional form for writing the activity coefficient of the macromolecular component in a three-component system (Scatchard, 1946; Johnson *et al.*, 1954, 1957; Casassa and Eisenberg, 1960, 1964; Eisenberg and Casassa, 1960). Since we do not know the molalities of the associating species *a priori*, it is more convenient to use weight/kg of solvent. The condition for electroneutrality of the solution *inside* the dialysis membrane is

$$W_X/M_X = zW_M/M_M + qW_D/M_D + W_B/M_B \quad (27)$$

Here M represents the monomer and D the dimer; also $M_D = 2M_M$. We assume that q, the charge on the dimer, is twice the charge on the monomer, i.e., $q = 2z$. Thus

$$W_X/M_X = zW_2/M_M + W_B/M_B \quad (27a)$$

The quantity W_2 is the total weight of macromolecule/kg of solvent: $W_2 = W_M + W_D$. For the *outer* solution, the electroneutrality condition is

$$W_X'/M_X = W_B'/M_B \quad (27b)$$

Primes are used to designate the outer solution.

The membrane distribution parameter, Γ, of Eisenberg and Casassa (1960) becomes

$$\Gamma = \left(\frac{W_B'}{M_B} - \frac{W_B}{M_B}\right) \Big/ \frac{zW_2}{M_M} \quad (28)$$

or

$$\Gamma\, zW_2/M_M = (W_B'/M_B) - (W_B/M_B) \quad (28a)$$

The concentration of the diffusible ions inside the membrane can also be expressed as

$$W_B/M_B = (W_B'/M_B) + (\nu_{2B}W_2/M_M) = (W_3'/M_3) + (\nu_{2B}W_2/M_M) \quad (29)$$

$$W_X/M_X = (W_X'/M_X) + (\nu_{2X}W_2/M_M) = (W_3'/M_3) + (\nu_{2X}W_2/M_M) \quad (29a)$$

When equation (29) is substituted into equation (28a), we find

$$\nu_{2B} = -\Gamma z \quad (30)$$

By using equations (27a), (27b), (28a), and (29a) we find

$$\nu_{2X} = (1 - \Gamma)z \quad (30a)$$

Thus, the redefined monomer is represented as

$$PX_z - (\Gamma z)BX$$

and the dimer as

$$[PX_z - (\Gamma z)BX]_2$$

and so on for the other associating species, if present. The membrane distribution parameter, Γ, has been defined by Eisenberg and Casassa (1960) in terms of an ideal Donnan equilibrium, using an effective charge iz for the macromolecular charge. Following their procedure one obtains for associating systems

$$\Gamma = \frac{i}{2}\left(1 - \frac{izW_2M_3}{4M_MW_3} + \ldots\right) \quad (31)$$

The quantity i is a factor by which the stoichiometric charge is altered by interactions of unspecified nature. When there is binding in excess of the stoichiometric macromolecular charge, then i may be negative. For neutral macromolecules i is zero; for macromolecules behaving as strong electrolytes i is one; for macromolecules without excess binding and behaving as weak electrolytes $0 < i < 1$.

One consequence of this redefinition of components (associating species) is that we can set $a_{23} = \partial \ln a_2/\partial W_3 = 0$, and we can write

$$d \ln a_{2M}^* = dW_{2M}^*/W_{2M}^* + \sum_i \frac{(\nu_{2i}^*)^2 dW_2^*}{M_M^*(W_i/M_i)} + \gamma_{22}^{0*} dW_2^* \quad (32)$$

In equation (32) the subscripts i refer to the ions B^+ or X^-. The asterisks indicate (Casassa and Eisenberg, 1960, 1964; Eisenberg and Casassa, 1960) that we are using the redefined component. Equation (32) becomes for the three-component, associating system

$$d \ln a_{2M}^* = dW_{2M}^*/W_{2M}^* + \frac{(\Gamma_2)^2 dW_2^*}{M_M^*\left(\frac{W_3}{M_3} - \frac{\Gamma z W_2^*}{M_M^*}\right)} + \frac{(1-\Gamma)^2 z^2 dW_2^*}{M_M^*\left(\frac{W_3}{M_3} + \frac{(1-\Gamma)zW_2^*}{M_M^*}\right)} + \gamma_{22}^{0*} dW_2^* \quad (32a)$$

In arriving at equation (32b) equations (30) and (30a) have been invoked. In dilute solutions equation (32a) may be rearranged as

$$d \ln a_{2M}^* = dW_{wM}^*/W_{2M}^* + \frac{(\Gamma z)^2 dW_2^*}{M_M^*\left(\frac{W_3}{M_3}\right)(1 + \ldots)}$$

$$+ \frac{(1 - \Gamma)z^2 dW_2^*}{M_M^* \left(\frac{W_3}{M_3}\right)(1 + \ldots)} + \gamma_{22}^{0*} dW_2^* \quad (32b)$$

In equations (32) to (32b) the activity coefficient of the monomer, γ (on the W concentration scale), is defined to be a function of the total amount of macromolecule present. Thus,

$$\ln \gamma_{2M}^* = f(W_2^*) = (d \ln \gamma_{2M}^*/dW_2^*)_{(W_2^*=0)} W_2^* + \ldots = \gamma_{22}^{0*} W_2^* + \ldots \quad (32c)$$

The condition for sedimentation equilibrium is that the total potential of the system be constant. If the total potential, $\bar{\mu}$, is represented by

$$\bar{\mu}_i = \mu_i - M_i \omega^2 r^2/2 \quad (33)$$

for component or species i, then at sedimentation equilibrium one has

$$d\mu_i = M_i(1 - \bar{v}_i\rho)\omega^2 d(r^2)/2 = ARTM_i \quad (33a)$$

Here,

$$d\mu_i = (\partial\mu_i/W_i)_{P,T} dW_i = RT \sum_j \frac{\partial \ln a_i}{\partial W_j} dW_j = RT \sum_j a_{ij} dW_j$$

and $A = (1 - \bar{v}_i\rho)\omega^2/2RT$. Thus we may rewrite equation (33a) as[2]

$$d \ln a_i = AM_i d(r^2)$$

$$d \ln a_i = \sum_j a_{ij} dW_j \quad (33b)$$

Invoking equation (32b) and (33b) one arrives at the sedimentation equilibrium equation for the monomer on the W concentration scale; thus one has

$$dW_{2M}^*/d(r^2) = \frac{W_{2M}^* M_M^* A^*}{1 + W_2^* \frac{dW_2^*}{dW_{2M}^*}[\phi^0(z) + \gamma_{22}^{0*}] + \ldots}$$

$$= \frac{W_{2M}^* M_M^* A^*}{1 + W_2^* \frac{dW_2^*}{dW_{2M}} \left[\frac{(\Gamma z)^2}{M_M^* \left(\frac{W_3}{M_3}\right)} + \frac{(1 - \Gamma)^2 z^2}{M_M^* \left(\frac{W_3}{M_3}\right)} + \gamma_{22}^{0*}\right]} \quad (33c)$$

$$A^* = (1 - \bar{v}^*\rho)\omega^2/2RT$$

$$\bar{v}^* = v^* \text{ monomer} = \bar{v}^* \text{ dimer}$$

[2] The convention used in equation (33b) is similar to one used by Johnson *et al.* (1954, 1958). With the redefinition of components equation (33b) becomes $d \ln a_2^* = a_{22}^* dW_2^*$.

In sedimentation equilibrium experiments it is more convenient to use concentrations on the $\hat{c}$ scale (g/ml) or the c scale (g/dl). The concentrations and concentration gradients are most generally measured by refractometric means in these experiments; in addition one uses the conservation of mass in order to calculate the concentration at any radial position. Sometimes absorption optics are used on the ultracentrifuge, and in this event concentrations are measured by photodensitometry. Thus, one must convert equation (33c) to the c scale; this may be done by the procedure proposed by Casassa and Eisenberg (1964). The relation between concentration on the $\hat{c}$ scale and concentration on the W scale is

$$\hat{c}_{2M}^* = W_{2M}^*/V_m \quad (34)$$

The quantity V_m is the volume of solution containing 1 kg of solvent; at constant temperature, V_m is a function of the composition of the system, as well as the pressure on the system. The quantity V_m can be written as

$$V_m = V_m^0 + (dV_m/dW_2^*)^0 W_2^* + \ldots \quad (34a)$$

The derivative, $(dV_m/dW_2^*)^\circ$, contains a compressibility term. One will find this derivative evaluated in the review of Casassa and Eisenberg (1964), as well as discussion on the quantity V_m and the conversion of concentration scales. If both sides of equation (33c) are multiplied by $(d\hat{c}_{2M}^*/dW_{2M}^*)_{sed}$, where

$$\left(\frac{d\hat{c}_{2M}^*}{dW_{2M}^*}\right)_{sed} = \frac{1}{V_m}\left[1 - \frac{W_{2M}^*}{V_m}\left(\frac{dV_m}{dW_2^*}\right)^0 \frac{dW_2^*}{dW_{2M}^*}\right] = \frac{1}{V_m}\left[1 - \hat{c}_{2M}^* \left(\frac{dV_m}{dW_2^*}\right)^0 \frac{dW_2^*}{dW_{2M}^*}\right] \quad (34b)$$

then one obtains

$$d\hat{c}_{2M}^*/d(r^2) = \frac{A^* M_M^* \left(\frac{W_{2M}^*}{V_m}\right)\left[1 - \hat{c}_{2M}^* \left(\frac{dV_m}{dW_2^*}\right)^0 \frac{dW_2^*}{dW_{2M}^*}\right]}{1 + W_2^* \frac{dW_2^*}{dW_{2M}^*}[\phi^0(z) + \gamma_{22}^{0*}]} \quad (35)$$

$$= \frac{A^* M_M^* \hat{c}_{2M}^*}{1 + \hat{c}_{2M}^* \frac{dW_2^*}{dW_{2M}^*}\left[V_m^0 \phi^0(z) + V_m^0 \gamma_{22}^* + \left(\frac{dV_m}{dW_2^*}\right)^0\right]}$$

It is a simple matter to convert to the c scale (g/dl) since $c = 100\hat{c}$. If one assumes, as was done for the case of neutral, associating macromolecules, that the activity coefficient of the associating species on the c scale can be expressed as

$$\ln y_i = iBM_M^* c^* + 0(c^{*2}), \quad i = 1 \text{ (monomer)}, 2 \text{ (dimer)} \ldots \quad (35a)$$

then one obtains

$$c^* = c_M^* + K_2 c_M^{*2} \quad (35b)$$

since

$$y_2^*/y_1^{*2} = 1 \quad (35c)$$

It should also be noted that

$$M_{w(c)}^*/M_M^* = c_M^*(1 + 2K_2 c_M^*) \quad (35d)$$

and also that

$$dW_2^*/dW_{2M}^* = 1 + dW_{2D}^*/dW_{2M}^* = 1 + 2K_2 c_M^* \times \frac{\left[1 + \frac{\hat{c}_M}{2V_m}\frac{dV_m^0}{dW_2^*}\frac{dW_2^*}{d\hat{c}_M}\right]}{\left[1 + \frac{\hat{c}_M}{V_m}\frac{dV_m^0}{dW_2^*}\frac{dW_2^*}{d\hat{c}_M}\right]} \cong 1 + 2K_2 c_M^* \quad (35e)$$

Utilizing equations (35a) to (35e), equation (35) becomes

$$dc_{2M}^*/d(r^2) = A^* M_M^* c_M^*/(1 + B^* M_{w(c)}^* c^*) \quad (36)$$

Since $dc^*/d(r^2) = (1 + 2K_2 c_{2M}^*)dc_{2M}^*/d(r^2)$, one obtains for the observed concentration gradient of the macromolecular solution the following equation

$$dc^*/d(r^2) = A^* c^* M_{w(c)}^*/(1 + B^* M_{w(c)}^* c^*) \quad (36a)$$

This equation can be rearranged to give

$$M_M^*/M_{w\ \mathrm{app}}^* = M_M^*/M_{w(c)}^* + B^* M_M^* c^* \quad (36b)$$

The quantities B^* and $M_{w\ \mathrm{app}}^*$ are defined as follows:

$$B^* = [V_m^0 \phi^0(z) + V_m^0 \gamma_{22}^{*0} + (dV_m/dW_2^*)^0]/100 M_M^* \quad (36c)$$

$$M_{w\ \mathrm{app}}^* = M_{w(c)}^*/(1 + B^* M_{w(c)}^* c^*) \quad (36d)$$

It follows then that one can apply the previous treatment to charged, associating macromolecules, provided one defines the associating species properly. As an alternative to the Eisenberg-Casassa (1960) definition of components, one could use the Scatchard-Bregman (1959) definition of components to obtain sedimentation equilibrium equations for associating systems. For nonassociating systems, Casassa and Eisenberg (1960, 1964) have discussed the applicability of sedimentation equilibrium equations and the effect of salt redistribution; these conditions are also applicable to sedimentation equilibrium in associating systems.

As a final remark, one notes that the theory presented here for a three-component system can be extended to multicomponent systems: water, an associating ionizable macromolecule, and two or more diffusible, supporting electrolytes. For this case one sets all the $a_{2K(\mathrm{diffusible})}$ equal to zero, and one again obtains an equation in exactly the same form as equation (36a) or (36b). Thus, if one had a four-component system: water (1), PX_z (2), BX (3), and HX (5), one defines the monomeric, associating species as

$$PX_z - \frac{m_3}{m_3 + m_5}\Gamma_{zBX} - \frac{m_5}{m_3 + m_5}\Gamma_{zHX}$$

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