Sedimentation Equilibrium in Reacting Systems. V. The Application of $\Sigma c_i M_i^2$ to the Analysis[*]

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ABSTRACT: In sedimentation equilibrium experiments on chemically reacting systems of the types $n\mathrm{P}_1 \rightleftarrows \mathrm{P}_n$, $n = 2, 3, \ldots$, or $n\mathrm{P}_1 \rightleftarrows q\mathrm{P}_2 + m\mathrm{P}_3 + \ldots$, the quantity $\Sigma c_i M_i^2$ can be obtained from the equation

$$\frac{-\Sigma c_i M_i^2}{M_1^2} \equiv \psi = \frac{\mathrm{d}\left(\dfrac{M_1}{cM_{\mathrm{w\,app}}}\right)\Big/\mathrm{d}c}{\left[\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1\right]^3}$$

The equations resulting from a combination of ψ with previously available relations can be used to evaluate the equilibrium constant or constants and the nonideal term, or they may be used to check calculations done with previously available equations. In analyzing these associating systems, one may encounter false hypotheses for the type of association; there are two suggested ways to treat this. One procedure is to use the equations that result from a combination of ψ with other available equations and see if these new equations will satisfy the association hypothesis. A second procedure is to generate a plot of $M_1/M_{\mathrm{w\,app}}$ *vs.* c for the false hypothesis and compare it with the observed $M_1/M_{\mathrm{w\,app}}$ *vs.* c plot. By the application of these techniques, one is in a better position to cast aside false hypotheses for the type of association. In addition, these techniques allow, in some cases, verification of previous calculations on the equilibrium constant or constants and the nonideal term. The application of these techniques to real (the association of α-chymotrypsin and lysozyme) and synthetic examples is shown.

In previous communications (Adams, 1965a,b) it has been shown how one can evaluate the number-average[1] ($M_{n(c)}$) or the apparent number-average ($M_{n\,\mathrm{app}}$) molecular weights and use them with other experimentally available quantities to evaluate K_i ($i = 2, 3 \ldots$), the equilibrium constant or constants, and BM_1, the nonideal term, for chemically reacting systems of the types

$$n\mathrm{P}_1 \rightleftarrows \mathrm{P}_n,\ n = 2,3\ldots \tag{1}$$

or

$$n\mathrm{P}_1 \rightleftarrows q\mathrm{P}_2 + m\mathrm{P}_3 \tag{2}$$

A subsequent paper (Adams and Filmer, 1966) has shown the results of applications of this method to real and synthetic examples. In this paper they noted that one could encounter false solutions which fortunately could be ruled out. The use of the quantity L ($L = K_2 - BM_1$), obtained, for example, from the limiting slope of a plot of $M_1/M_{\mathrm{w\,app}}$ against c, makes it easy to rule out false solutions, but it appears that the evaluation of the quantity L could be quite difficult because of experimental error in the vicinity of zero concentration of the macromolecule. The need clearly exists for a method of checking the calculations at real concentrations and removing the need for trying to obtain the quantity L. Such a method appears to be found if one uses the quantity $-\Sigma c_i M_i^2/M_1^2$, which will be called ψ; this quantity (ψ) can be obtained from the experimental data used in analyzing the associating systems described by eq 1 and 2. The method for obtaining ψ will be described, and the application of this quantity to real and synthetic examples will be shown.

The Procedure for Obtaining $\Sigma c_i M_i^2$

Background Information. It will be assumed in the treatment that follows that previously stated (Adams and Fujita, 1963; Adams and Williams, 1964; Adams 1965a,b) conditions apply: (1) the partial specific volumes of all associating species are equal, (2) the refractive index increments of all associating species are equal; and (3) the logarithm of the activity coefficient of each associating species i can be represented by

$$\ln y_i = iBM_1c + 0(c^2) \tag{3}$$

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[1] All average molecular weights of chemically reacting systems described by eq 1 and 2 are concentration dependent. To indicate that one is dealing with such systems (and to distinguish these systems from nonreacting systems), it is convenient to use the symbols $M_{n(c)}$, $M_{w(c)}$, $M_{z(c)}$, to represent these concentration-dependent average molecular weights. This is a convention suggested by Adams and Williams (1964).

Using molar chemical potentials, μ_i, the condition for chemical equilibrium for the associations described by eq 1 is

$$n\mu_1 = \mu_n,\ n = 2, 3\ldots \tag{3a}$$

and this equilibrium condition is the same for the association described by eq 2. These associating systems are known as self-associating systems. As a consequence of eq 3 one notes that the total concentration is given by

$$c = c_1 + K_n c_1^n,\ n = 2, 3\ldots \tag{3b}$$

for the monomer–n-mer equilibrium, the type described by eq 1. For the monomer–dimer–trimer equilibrium, the type described by eq 2, the total concentration is given by

$$c = c_1 + K_2 c_1^2 + K_3 c_1^3 \tag{3c}$$

Equations similar to eq 3c can be developed for associations involving monomer, dimer, and n-mer or n-mers. It should be noted here that as a result of eq 3 one observes that

$$c_n = K_n c_1^n,\ n = 2, 3\ldots \tag{3d}$$

since

$$y_n/y_1^n = 1,\ n = 2, 3\ldots \tag{3e}$$

It should be emphasized that eq 3e does not mean that y_1 or y_n is necessarily one at all concentrations; it only shows that the ratio given in eq 3e is always one. Whenever eq 3 applies one also notes that the reduced apparent weight-average ($M_1/M_{\mathrm{w\,app}}$) molecular weight is defined by

$$\frac{M_1}{M_{\mathrm{w\,app}}} = \frac{M_1}{M_{\mathrm{w}(c)}} + BM_1c \tag{4}$$

Here

$$M_{\mathrm{w}(c)} = M_{\mathrm{w}r} = \Sigma c_{ir}M_i/c_r \tag{4a}$$

The quantity $M_{\mathrm{w\,app}}$ is defined by the equation

$$\frac{1}{A}\frac{\mathrm{d}\ln c_r}{\mathrm{d}(r^2)} = M_{\mathrm{w\,app}} = \frac{M_{\mathrm{w}(c)}}{(1 + BM_{\mathrm{w}(c)}c)} \tag{4b}$$

where $c = c_r$ = total concentration at any radial position. The quantity A is defined as $A = (1 - \bar{v}\rho)\omega^2/2RT$, where the quantities in the term A have their usual meaning (Lansing and Kraemer, 1935; Svedberg and Pedersen, 1940; Schachman, 1959; Fujita, 1962). It has also been shown by Adams and Williams (1964) that one can obtain the apparent weight fraction (f_a) of the monomer by the equation

$$\ln f_a = \int_0^c \left(\frac{M_1}{M_{\mathrm{w\,app}}} - 1\right)\frac{\mathrm{d}c}{c} = \ln f + BM_1c \tag{4c}$$

where $f = c_1/c$ = the weight fraction of monomer. From the quantity f_a it may be noted that

$$\alpha \equiv cf_a = c_1e^{BM_1c} \tag{4d}$$

More recently it has been shown (Adams, 1965a) that one can obtain the apparent number-average ($M_{n\,\mathrm{app}}$) molecular weight from a series of sedimentation equilibrium experiments at different initial concentrations by the use of the equation

$$\frac{cM_1}{M_{n\,\mathrm{app}}} = \int_0^c \frac{M_1}{M_{\mathrm{w\,app}}}\mathrm{d}c = \frac{cM_1}{M_{n(c)}} + \frac{BM_1c^2}{2} \tag{4e}$$

An ideal solution will be defined here as a solution in which BM_1, the nonideal term or virial coefficient, is zero for all concentrations of the associating solute. For ideal solutions the apparent values of the average molecular weights are replaced by their true values, and in this case eq 4e gives the number-average ($M_{n(c)}$) molecular weight.

It must be emphasized that $M_{n\,\mathrm{app}}$, the apparent number-average molecular weight, is evaluated from a series of sedimentation equilibrium experiments at different initial concentrations. The $M_1/M_{\mathrm{w\,app}}$ data from these experiments are plotted against the total concentration c ($c = c_r$), and the resulting curve is extrapolated to zero concentration of the associating solute, where $M_1/M_{\mathrm{w\,app}} = 1$. The area under the resulting curve, from a plot of $M_1/M_{\mathrm{w\,app}}$ against c, between $c = 0$ and $c = c$ gives $cM_1/M_{n\,\mathrm{app}}$.

Previously it has been shown (Adams and Filmer, 1966) for chemically reacting systems described by eq 1 and 2 that

$$M_{z(c)} = \frac{\mathrm{d}(cM_{\mathrm{w}(c)})}{\mathrm{d}c} \tag{5}$$

for ideal solutions, and for nonideal solutions they observed that

$$M_{z\,\mathrm{app}} = \frac{\mathrm{d}(cM_{\mathrm{w\,app}})}{\mathrm{d}c} = \frac{M_{z(c)}}{(1 + BM_{\mathrm{w}(c)}c)^2} \tag{5a}$$

The quantity $M_{z(c)}$ is defined as

$$M_{z(c)} = \frac{\Sigma c_iM_i^2}{\Sigma c_iM_i} = \frac{\Sigma c_iM_i^2}{cM_{\mathrm{w}(c)}} \tag{5b}$$

Equation 5b was first stated by Lansing and Kraemer (1935) for nonreacting systems. It has been noted by Adams and Filmer (1966) that $M_{z(c)}$ or $M_{z\,\mathrm{app}}$ as such were not very useful in the analysis of these chemically reacting systems. On the other hand the quantity $\psi = -\Sigma c_iM_i^2/M_1^2$ will turn out to be quite useful in this analysis, and it will be shown how to obtain ψ with ideal and nonideal systems. Furthermore, it will be shown how the quantity ψ can be used with $M_{w(c)}$, $M_{n(c)}$, or their apparent values, and other available quantities to evaluate BM_1, K_2, etc.

Derivation of the Equation for ψ. With ideal solutions eq 5b can be rearranged to give

$$cM_{w(c)}M_{z(c)} = \Sigma c_i M_i^2 = \frac{1}{2}\frac{d(cM_{w(c)})^2}{dc} \qquad (6)$$

Equation 6 may be divided by M_1^2 to give

$$cM_{w(c)}M_{z(c)}/M_1^2 = \Sigma i^2 K_i c_1^i = -\psi \qquad (6a)$$

where $i = 1, 2, \ldots, M_i = iM_1$, and $K_1 = 1$. For an ideal monomer–dimer–trimer association, eq 6 can be written as

$$cM_{w(c)}M_{z(c)}/M_1^2 = -\psi = c_1 + 4K_2c_1^2 + 9K_3c_1^3 \quad (6b)$$

It is convenient to define a K_1 which is identically equal to one;[2] this allows one to write the right-hand side of eq 6a as a simple summation. Although eq 6 or 6a can be used with ideal solutions, they cannot be applied at present to nonideal systems to obtain $\Sigma c_i M_i^2$. In order to obtain $\Sigma c_i M_i^2$ with nonideal systems one uses the relation

$$\frac{d}{dc}\left(\frac{M_1}{cM_{w\,app}}\right) = \frac{-M_1M_{z\,app}}{(cM_{w\,app})^2} = \frac{-M_1M_{z(c)}}{(1 + BM_{w(c)}c)^2}\,\frac{(1 + BM_{w(c)}c)^2}{(cM_{w(c)})^2} = \frac{-M_1M_{z(c)}}{(cM_{w(c)})^2} = \frac{-\Sigma i^2K_ic_1^i}{\Sigma(iK_ic_1^i)^3} \qquad (6c)$$

$$K_1 \equiv 1$$

Although eq 6c can be written in terms of c, $M_{w\,app}$, and $M_{z\,app}$, it is not necessary to evaluate $M_{z\,app}$ to obtain this equation.[3] Having available eq 6c, we now proceed to use it in the general equation for ψ. It is noted from eq 6c that

$$(\Sigma iK_ic_1^i)^3\frac{d(M_1/cM_{w\,app})}{dc} = \Sigma i^2K_ic_1^i \qquad (7)$$

For eq 7 to be useful in the analysis, it is necessary to find an expression for $\Sigma iK_ic_1^i$, and this can be done with the aid of eq 4, since it is noted that

$$\frac{M_1}{M_{w\,app}} = \frac{c}{\Sigma iK_ic_1^i} + BM_1c \qquad (8)$$

On dividing eq 8 by c, and subsequently subtracting the quantity BM_1 from both sides, it is observed that

$$\frac{M_1}{cM_{w\,app}} - BM = \frac{1}{\Sigma iK_ic_1^i} \qquad (8a)$$

The reciprocal of eq 8a gives the desired result, namely[4]

$$\frac{1}{\dfrac{M_1}{cM_{w\,app}} - BM_1} = \Sigma iK_ic_1^i \qquad (8b)$$

Applying eq 8b to eq 7 leads to the result that

$$\frac{d(M_1/cM_{w\,app})/dc}{\left(\dfrac{M_1}{cM_{w\,app}} - BM_1\right)^3} = -\Sigma i^2K_ic_1^i \equiv \psi \qquad (8c)$$

Equation 8c is a general equation for ψ, since it can be used with ideal or nonideal solutions of self-associating solutes. We can combine ψ with some of the previously described quantities, such as those given in eq 3b or 3c, 4, 4d, and 4e, to develop equations for evaluating the equilibrium constant or constants and the nonideal term.

Analysis of Self-Associating Systems

The Monomer–Dimer–Trimer Association. For the monomer–dimer–trimer association it is observed that

$$\frac{cM_1}{M_{n\,app}} = c_1 + \frac{K_2c_1^2}{2} + \frac{K_3c_1^3}{3} + \frac{BM_1c^2}{2} \qquad (9)$$

$$1\Big/\left(\frac{M_1}{cM_{w\,app}} - BM_1\right) = c_1 + 2K_2c_1^3 + 3K_3c_1^3 \quad (9a)$$

and that eq 8c becomes

$$\psi = -c_1 - 4K_2c_1^2 - 9K_3c_1^3 \qquad (9b)$$

Recalling from eq 4d that $c_1 = \alpha e^{-BM_1c}$ and using eq 3c, 9, 9a, and 9b one obtains

$$\frac{12cM_1}{M_{n\,app}} - 4c = 6c_1 + 6BM_1c^2 + \psi +$$

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[2] The procedure was suggested by the late Dr. F. E. LaBar.

[3] If for some reason it is desired to evaluate $M_{z\,app}$, eq 5a could be used. In addition the following equation can be used, namely

$$M_{z\,app} = \frac{1}{A}\,\frac{d\ln\left(\dfrac{1}{r}\dfrac{dc}{dr}\right)}{d(r^2)}$$

This last equation has been used by Holmberg *et al.* (1956) and by Johnson and Kraus (1956) to obtain M_{zr} in studies on polydisperse, nonassociating solutes.

[4] With a monomer–dimer–trimer association as an example one notes that eq 8a becomes

$$\frac{M_1}{cM_{w\,app}} - BM_1 = \frac{1}{c_1 + 2K_2c_1^2 + 3K_3c_1^3}$$

$$\frac{3}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} = 6\alpha e^{-BM\alpha c} + 6BM_1c^2 + \psi + \frac{3}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} \quad \text{(9c)}$$

Equation 9 can be solved for BM_1 by successive approximations of the unknown, BM_1. Knowing the value of BM_1, the monomer concentration c_1 can be obtained from eq 4d or from other equations, such as eq 9c itself, since $6c_1 = 6\alpha e^{-BM_1c}$. One can obtain K_2 from the following equation

$$\frac{3}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} + \psi = 2c_1 + 2K_2c_1^2 \quad \text{(9d)}$$

since BM_1 and c_1 are known. Knowing BM_1, c_1, and K_2, the quantity K_3 is obtained from eq 3c, 9, 9a, or 9b. One obtains $M_{\mathrm{w}(c)}$ from eq 4 and $M_{n(c)}$ from eq 4e. Other procedures for evaluating c_1, K_2, K_3, $M_{\mathrm{w}(c)}$, and $M_{n(c)}$ have been described by Adams (1965a) and Adams and Filmer (1966). An alternative equation that can be applied to the monomer–dimer–trimer association is

$$\frac{30cM_1}{M_{n\,\mathrm{app}}} - 19c = 12c_1 + \psi + 15BM_1c^2 = 12\alpha e^{-BM_1c} + \psi + 15BM_1c^2 \quad \text{(9e)}$$

Here eq 3c, 4d, 9, and 9b were used to obtain eq 9e. Equation 9e is solved by successive approximations of the unknown, BM_1; the other quantities are evaluated by the procedures described after eq 9c. One virtue of using $\psi = -(\Sigma c_iM_i^2)/M_1^2$ in the analysis is that this method is applicable at real concentrations of the associating macromolecule; it definitely avoids the use of a limiting slope from a plot of $M_1/M_{\mathrm{w\,app}}$ against c to obtain the quantity $L = K_2 - BM_1$ and the subsequent use of L to obtain K_2, etc. On the other hand, this method is subject to adverse criticism because (1) differentiation is a potential error-producing process, and (2) only one virial coefficient has been used. For some nonideal solutions other virial coefficients, the third, etc.,[5] could affect the results; this difficulty could be overcome in some instances by using two virial coefficients in the definition of the activity coefficient, as has been done previously (Adams, 1965a). At present no treatment involving two virial coefficients will be included. With regard to the first objection, this appears to be a fact of life that would have to be endured unless other relations for analyzing these systems are discovered. It should be emphasized that ψ is not known *a priori;* it (ψ) must be evaluated for each choice of BM_1 in the successive approximations.

The present analysis can be extended to include tetramer; in this case eq 9, 9a, 9b, and 3c would have to include a $K_4c_1^4$ term.[6] For the monomer–dimer–trimer–tetramer association one observes that

$$\frac{24cM_1}{M_{n\,\mathrm{app}}} - 26c = 6c_1 + 12BM_1c^2 + \psi - \frac{9}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} \quad \text{(10)}$$

$$= 6\alpha e^{-BM_1c} + 12BM_1c^2 + \psi - \frac{9}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1}$$

Equation 10 is obtained in a manner similar to the one used to obtain eq 9c and 9e; it is also solved for BM_1 by successive approximations of the unknown, BM_1. The other quantities c_1, K_2, etc., are evaluated in a manner similar to the one described after eq 9c for the monomer–dimer–trimer association.

The Monomer–n-Mer Association. For the monomer–n-mer ($n = 2, 3 \ldots$) association, one observes that

$$\psi = -c_1 - n^2K_nc_1^n \quad \text{(11)}$$

$$\frac{cM_1}{M_{n\,\mathrm{app}}} = c_1 + \frac{K_nc_1^n}{n} + \frac{BM_1c^2}{2} \quad \text{(11a)}$$

and that

$$\frac{1}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} = c_1 + nK_nc_1^n \quad \text{(11b)}$$

Let us consider the situation where n is known; here eq 4d can be used in various combinations with eq 3b, 11, 11a, and 11b to give

$$\frac{ncM_1}{M_{n\,\mathrm{app}}} - c = \psi + \frac{n}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} + \frac{nBM_1c^2}{2} \quad \text{(11c)}$$

or alternatively to give

$$(n+1)\left[n\left(\frac{cM_1}{M_{n\,\mathrm{app}}}\right) - c\right] - n^2c = \psi + \frac{n(n+1)BM_1c^2}{2} \quad \text{(11d)}$$

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[5] It is customary to refer to BM_1 as the second virial coefficient.

[6] For example, with a monomer–dimer–trimer–tetramer association one notes that

$$\frac{1}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} = c_1 + 2K_2c_1^2 + 3K_3c_1^3 + 4K_4c_1^4$$

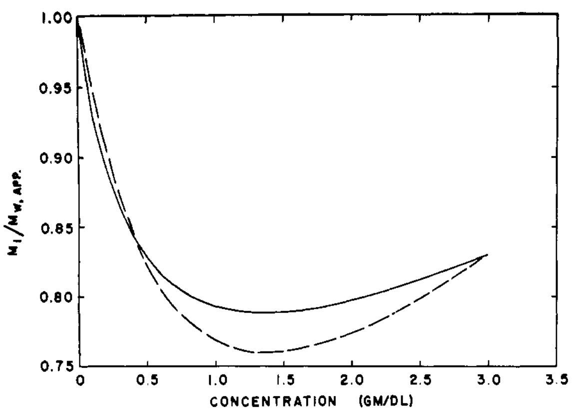

FIGURE 1: Synthetic example. This figure shows how one may use comparison plots of $M_1/M_{w\ app}$ *vs.* c. If this were a real experiment the observed data and the plot for the monomer–dimer–trimer (dashed line) equilibrium would coincide. The plot for the monomer–dimer association (solid line) intersects the other curve at c = 3.00 and 0.40 g/dl. At c = 3.00 one will encounter the possibility of a monomer–dimer association in the successive approximations to decide what association is present; this is caused by the intersection of the two curves. By generating the $M_1/M_{w\ app}$ *vs.* c plot for the monomer–dimer association, one can show that it does not fit the observed plot of $M_1/M_{w\ app}$ *vs.* c (in this case the curve for the monomer–dimer–trimer), and hence does not describe the type of association present.

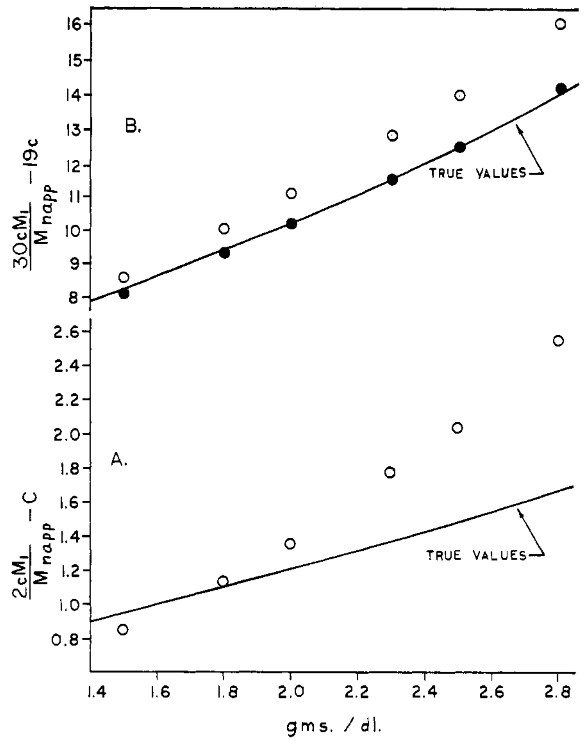

FIGURE 2: Synthetic example. (A) This figure shows the results obtained from eq 12 with the false monomer–dimer (open circles). The true values of $(2cM_1/M_{n\ app}) - c$ are given by the solid line, and it can be seen that the false monomer–dimer is spurious. (B) This figure shows the results obtained when the false monomer–dimer (open circles) and the true monomer–dimer–trimer (filled circles) data are used in eq 9e. The true values of $(30cM_1/M_{n\ app}) - 19c$ are given by the solid curve.

Equations 11c or d can be solved for BM_1 by successive approximations of the unknown, BM_1. Knowing BM_1, the quantities c_1, K_n, etc., can be evaluated by using methods similar to those described after eq 9c, or by using previously described methods (Adams, 1965a; Adams and Filmer, 1966). If n is unknown, one can develop equations that allow the evaluation of BM_1, and subsequently $n, K_n, M_{w(c)}$, and $M_{n(c)}$; these methods have been described previously (Adams, 1965a).

Tests with Real and Synthetic Examples

Previously in the analysis of a synthetic, nonideal monomer–dimer–trimer association, it was noted that one could encounter a false solution (Adams and Filmer, 1966),[7] which indicated a nonideal, monomer–dimer association would satisfy the data. There are essentially two ways of casting aside false solutions. One way, which was suggested previously (Adams and Filmer, 1966), is to make a plot of the observed $M_1/M_{w\mathrm{app}}$ *vs.* c data, and compare this plot with a corresponding plot obtained from the suspected false solution data. This kind of plot is shown in Figure 1; here it is noted that the "false" monomer–dimer association plot intersects the "true" monomer–dimer–trimer association plot at c = 3.00 g/dl. In addition it may also be noted that the "false" monomer–dimer association does not fit the "true" data points, *i. e.*, the curve (of $M_1/M_{w\mathrm{app}}$ *vs.* c) in this case for the monomer–dimer–trimer association. On this basis one can clearly show that the monomer–dimer association is a false solution.

The other approach to casting aside false solutions that can be used with a monomer–dimer association is to use the equations

$$\frac{2cM_1}{M_{n\mathrm{app}}} - c = \psi + \frac{1}{\dfrac{M_1}{cM_{w\mathrm{app}}} - BM_1} + BM_1c^2 \quad (12)$$

and

$$\frac{6cM_1}{M_{n\mathrm{app}}} - 7c = \psi + 3BM_1c^2 \quad (12a)$$

Figure 2A displays the results obtained with eq 12; it is apparent from this figure that the monomer–dimer association is indeed false. If a monomer–dimer association is correct, it will satisfy the equations for a mono-

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[7] See Table III of their paper.

mer–dimer–trimer association; here use can be made of eq 9c and 9e. In addition the following equation can be used for a monomer–dimer–trimer association (Adams, 1965a; Adams and Filmer, 1966)

$$\frac{6cM_1}{M_{n\,\mathrm{app}}} - 5c = 2c_1 + 3BM_1c^2 - \frac{1}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1} \quad (12b)$$

$$= 2\alpha e^{-BM_1c} + 3BM_1c^2 - \frac{1}{\dfrac{M_1}{cM_{\mathrm{w\,app}}} - BM_1}$$

Figure 2B shows the results obtained with eq 9e, and it confirms the falseness of the monomer–dimer association. Similar results have been obtained with eq 9c and 12b. Thus, by making comparison plots of $M_1/M_{\mathrm{w\,app}}$ against c, and by the use of additional equations, some of which involve the quantity ψ, it can be shown that the monomer–dimer association is false for this example.

With the α-chymotrypsin data of Rao and Kegeles (1958), it was noted that two possibilities occurred: a monomer–dimer–trimer (which Rao and Kegeles favored) and a monomer–dimer–trimer–tetramer association. The calculated plots of $M_1/M_{\mathrm{w\,app}}$ against c for the two examples were quite close to the observed curve of $M_1/M_{\mathrm{w\,app}}$ *vs.* c; in this case any differences between the two calculated curves (of $M_1/M_{\mathrm{w\,app}}$ *vs.* c) and the observed curve would be better displayed by difference plots. The difference plots between the observed and calculated values of $M_1/M_{\mathrm{w\,app}}$ at various concentrations are displayed in Figure 3, and it would appear that both curves give good agreement. Here one may have an analogy to what McCracken and Dorn (1964) refer to as "ill-conditioned" equations, and the resolution of this problem is not easy. Table I gives a comparison of tests on both possibilities using eq 10 for the monomer–dimer–trimer–tetramer association, and it would appear that within experimental error both choices are possible. In addition, tests with eq 9c and 9e are shown in Figure 4; these tests indicate that the monomer–dimer–trimer association is also a good choice. Other tests with eq 12b for a monomer–dimer–trimer association which have

TABLE I: Tests on the Rao and Kegeles (1958) Data on α-Chymotrypsin Using Eq 10 for a Monomer–Dimer–Trimer–Tetramer Association.

	$(24cM_1/M_{n\,\mathrm{app}}) - 26c$		
c	Obsd	Calcd for Monomer–Dimer–Trimer[a]	Monomer–Dimer–Trimer–Tetramer[b]
1.5	−14.2	−14.5	−14.4
2.0	−20.9	−21.4	−21.3
2.3	−25.2	−25.0	−24.5
2.5	−28.0	−28.6	−28.3
2.7	−31.0	−31.1	−30.4

[a] For the monomer–dimer–trimer association $\overline{BM_1} = -0.018$, $\overline{K}_2 = 0.89$, and $\overline{K}_3 = 0.92$. [b] For the monomer–dimer–trimer–tetramer association $\overline{BM_1} = 0.004$, $\overline{K}_2 = 1.08$, $\overline{K}_3 = 0.27$, and $\overline{K}_4 = 0.88$.

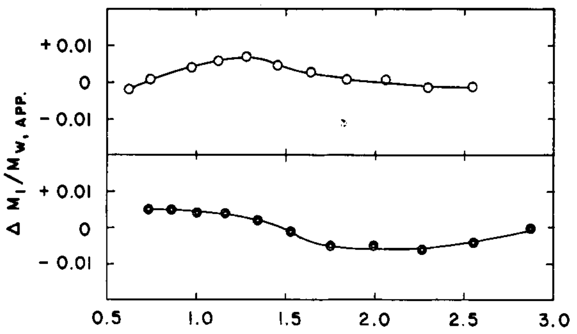

FIGURE 3: α-Chymotrypsin (Rao and Kegeles, 1958). This figure shows the result of difference plots between the calculated and observed values of $M_1/M_{\mathrm{w\,app}}$ at various concentrations for the two possible types of association. It is evident from the graphs that both possibilities, the monomer–dimer–trimer (upper curve) and the monomer–dimer–trimer–tetramer (lower curve) association, are good choices.

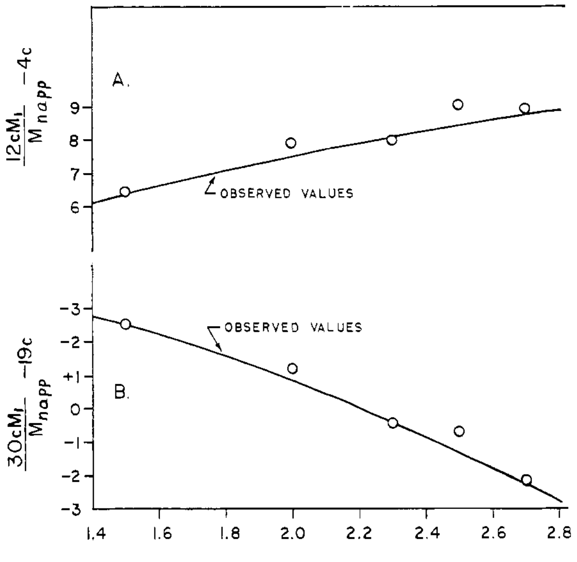

FIGURE 4: α-Chymotrypsin (Rao and Kegeles, 1958). (A) Tests with eq 9c for a monomer–dimer–trimer association. (B) Tests with eq 9e for a monomer–dimer–trimer association.

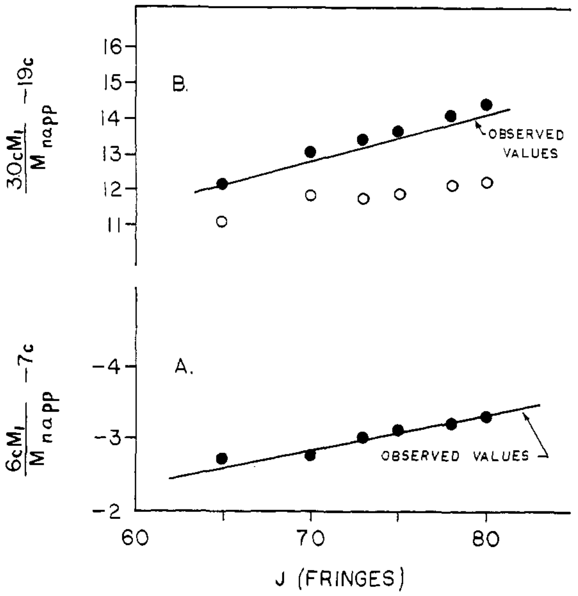

FIGURE 5: Lysozyme (Adams and Filmer, 1966). (A) Tests with eq 12a for the monomer–dimer association. The observed values of $(6cM_1/M_{n\,\mathrm{app}}) - 7c$ are given by the solid line and the calculated values of $(6cM_1/M_{n\,\mathrm{app}}) - 7c$ by the filled circles. (B) Tests with eq 9e for a monomer–dimer–trimer association. The observed values of $(30cM_1/M_{n\,\mathrm{app}}) - 19c$ are given by the solid line, and the calculated values are given by the filled circle for the monomer–dimer and the open circles for the monomer–trimer.

given good agreement are recorded elsewhere (Adams and Filmer, 1966). Thus we confirm the observation by Rao and Kegeles that two possibilities might exist for this association. Some of this discrepancy in the analysis may be due to temperature effects. At the time Rao and Kegeles (1958) did their experiments, they did not have an accurate temperature control system available. By doing these experiments at two different temperatures where the association occurs, it may be possible to resolve these difficulties, since one can see which type of association is more consistent with the experimentally observed data at the two temperatures.

The third example to be considered is the association of lysozyme at 25° (Adams and Filmer, 1966). Although it was fairly well established that a monomer–dimer association described the experimental data, a false monomer–trimer association was, nevertheless, encountered in the successive approximations. Figure 5A shows the results obtained with eq 12a for a monomer–dimer association, and it is seen that the fit of the calculated to the observed data is excellent. Although not shown, results similar to those shown in Figure 5A have been obtained with eq 12. Figure 5B shows the results obtained with eq 9e when applied to a monomer–dimer and a monomer–trimer association; it is quite evident that the monomer–dimer association gives much better agreement. The value of BM_1 used in these calculations is an averaged value, and this may account for the slight variation between the calculated and observed values. The application of eq 9c gives results (not shown here) that are in accord with the results obtained from Figure 5B. The monomer–dimer ($K_3 = 0$) and the monomer–trimer ($K_2 = 0$) associations can be thought of as special cases of the monomer–dimer–trimer association; thus the proper choice for the association should satisfy the equations for the monomer–dimer–trimer association. The results obtained by these tests and displayed in Figure 5A,B confirm the presence of a monomer–dimer association for lysozyme at 25° in 0.15 M NaCl and 0.01 M sodium phosphate buffer at pH 6.7.

In this presentation it has been shown how the quantity ψ may be used to evaluate BM_1, K_2, etc. This technique will be applicable to sedimentation equilibrium experiments on chemically reacting systems described by eq 1 and 2, and quite likely it may be applicable to light-scattering[8] experiments on these systems. When Rayleigh and schlieren optics are used to obtain $M_{w\,\mathrm{app}}$, only one numerical differentiation of the data is required for the evaluation of $\mathrm{d}(M_1/cM_{w\,\mathrm{app}})/\mathrm{d}c$ in eq 6c, from which the quantity ψ is obtained. While this technique is applicable in principle to osmotic pressure experiments on these chemically reacting systems, it would be necessary to do a second differentiation to obtain $\mathrm{d}(M_1/cM_{w\,\mathrm{app}})/\mathrm{d}c$. It should be recalled that $M_1/M_{w\,\mathrm{app}} = \mathrm{d}(cM_1/M_{n\,\mathrm{app}})/\mathrm{d}c$ for osmotic pressure experiments (Adams, 1965b) on these chemically reacting systems. Since differentiation is a potential error-producing process, one is quite likely to acquire more error with a second differentiation, and this fact would make the application of the quantity ψ to the analysis of osmotic pressure data questionable. Similar considerations would apply to the analysis of sedimentation equilibrium data obtained exclusively from Rayleigh optics.

For the examples in this discussion, the quantity $\mathrm{d}(M_1/cM_{w\,\mathrm{app}})/\mathrm{d}c$ has been obtained numerically from Stirling's interpolation formula. A brief explanation of this method will be given; more complete details will be found in the texts by Fröberg (1965) and by Scarborough (1962). In order to obtain the derivative we must make a smooth plot of $M_1/cM_{w\,\mathrm{app}}$ *vs.* c; from this plot we construct Table II. Here we have used the data from Figure 1. Essentially this method involves estimating the slope on either side of the desired point from first differences and then averaging the result. For very slowly changing functions larger intervals in c could be used, whereas smaller intervals in c could be used with rapidly chang-

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[8] Steiner (1953a,b) has postulated that there may be an association fluctuation coupling in light scattering of systems in rapid, dynamic equilibria; the result of this coupling would be the observation of too large a molecular weight ($M_{w\,\mathrm{app}}$). The validity of his assertions is a moot point at present. While some differences in the results of sedimentation equilibrium (Adams and Fujita, 1963; Jeffrey and Coates, 1963, 1966) and light scattering (Doty and Myers, 1953) of insulin have been noted, these differences may possibly be due to adsorption of the protein on the ultracentrifuge cell wall, or to solubility of the protein in the layering oil, or to other effects. It is hoped to resolve this problem in the near future.

TABLE II: Synthetic Example. Data used in the Evaluation of $d(M_1/cM_{w\,app})/dc$ and ψ for a Monomer–Dimer–Trimer Association.[a]

	$(M_1/cM_{w\,app}) = y$	Δy First Difference	ψ	
c (g/dl)			Calcd	True
1.7	0.4494[b]			
		−0.0233		
1.8	0.4261		−6.35	−6.35
		−0.0208		
1.9	0.4053			
		−0.0183		
2.0	0.3870		−7.36	−7.33
		−0.0165		
2.1	0.3705			

$$d(M_1/cM_{w\,app})/dc = \frac{1}{\Delta c}\left[\frac{\Delta y_{-1} + \Delta y_0}{2}\right]$$

$$\psi = d(M_1/cM_{w\,app})\bigg/\left[\frac{M_1}{cM_{w\,app}} - BM_1\right]^3$$

at $c = 2.0$

$$d(M_1/cM_{w\,app}) = \frac{1}{0.1}\left[\frac{-0.0183 - 0.0165}{2}\right] = -0.174$$

$$\psi = \frac{-0.174}{(0.2870)^3} = -7.36$$

[a] For this example $K_2 = 0.65$, $K_3 = 0.50$, and $BM_1 = 0.10$. [b] The data used in calculating $M_1/cM_{w\,app}$ are taken from the data used to make the dashed curve in Figure 1.

ing functions. The derivative needed for eq 6b could also be obtained from a least-squares polynomial fitted through the experimental data; Jeffrey and Coates (1966) have used this procedure to obtain $M_{w\,app}$ from Rayleigh optical data in their studies on the sedimentation equilibrium of insulin. It should be emphasized that a smoothed curve through the data is used in the analysis of these systems; the quantity $cM_1/M_{n\,app}$, for example, would be evaluated from a smoothed curve drawn through the plot of $M_1/M_{w\,app}$ against c.

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References

Adams, E. T., Jr. (1965a), *Biochemistry 4*, 1646.

Adams, E. T., Jr. (1965b), *Biochemistry 4*, 1655.

Adams, E. T., Jr., and Filmer, D. L. (1966), *Biochemistry 5*, 2971.

Adams, E. T., Jr., and Fujita, H. (1963), *Ultracentrifugal Anal. Theory Expt. Conf. Rockefeller Inst., New York, N. Y., 1962*, 119.

Adams, E. T., Jr., and Williams, J. W. (1964), *J. Am. Chem. Soc. 86*, 3454.

Doty, P., and Myers, G. E. (1953), *Discussions Faraday Soc. 13*, 51.

Fröberg, C. E. (1965), Introduction to Numerical Analysis, Reading, Mass., Addison-Wesley, Chapter 9.

Fujita, J. (1962), Mathematical Theory of Sedimentation Analysis, New York, N. Y., Academic, Chapters I and V.

Holmberg, R. W., Kraus, K. A., and Johnson, J. S. (1956), *J. Am. Chem. Soc. 78*, 5506.

Jeffrey, P. D., and Coates, J. H. (1963), *Nature 197*, 1004.

Jeffrey, P. D., and Coates, J. H. (1966), *Biochemistry 5*, 489.

Johnson, J. S., and Kraus, K. A. (1956), *J. Am. Chem. Soc. 78*, 3937.

Lansing, W. D., and Kraemer, E. O. (1935), *J. Am. Chem. Soc. 57*, 1369.

McCracken, D. D., and Dorn, W. S. (1964), Numerical Methods and Fortran Programming, New York, N. Y., Wiley, p 229.

Rao, M. S. N., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.

Scarborough, J. B. (1962), Numerical Mathematical Analysis, 5th ed, Baltimore, Md., Johns Hopkins, pp 133–135.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic, p 259.

Steiner, R. F. (1953a), *Arch. Biochem. Biophys. 44*, 121.

Steiner, R. F. (1953b), *Arch. Biochem. Biophys. 47*, 59.

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford, Clarendon, p 48.