Lee, C. C., and Samuels, E. R. (1964), *Can. J. Chem. 42*, 168.
Matsuo, T. (1964), *Bull. Chem. Soc. Japan 37*, 1844.
Mustafa, A., Asker, W., Khattab, S., and Zayed, S. (1961), *J. Org. Chem. 26*, 787.
Nakanishi, K. (1962), Infrared Absorption Spectroscopy, San Francisco, Calif., Holden-Day, p 47.
Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill, pp 132–138, 336.
Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W. (1964), *Biochem. J. 91*, 589.
Smyth, D. G., Nagamatsu, A., and Fruton, J. S. (1960), *J. Am. Chem. Soc. 82*, 4600.
Tawney, P. O., Snyder, R. H., Conger, R. P., Liebrand, K. A., Stiteler, C. H., and Williams, A. R. (1961), *J. Org. Chem. 26*, 15.
Uno, T., and Machida, K. (1962), *Bull. Chem. Soc. Japan 35*, 276.

Sedimentation Equilibrium in Reacting Systems. IV. Verification of the Theory*

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ABSTRACT: The theories [Adams, E. T., Jr., and Williams, J. W. (1964), *J. Am. Chem. Soc. 86*, 3454; Adams, E. T., Jr., (1965), *Biochemistry 4*, 1646] previously developed for nonideal associating systems have been verified by synthetic examples. The effects of variation in the virial coefficients or equilibrium constants on the association have been described. Details of the methods for evaluating $M_1/M_{n\,app}$ and $c_1e^{BM_1c}$ from the experimental data have been described.

The data of Rao and Kegeles [Rao, M. S. N., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724] on α-chymotrypsin have been examined, and it has been shown that one can do the analysis without recourse to additional sedimentation velocity experiments and the subsequent application of the Gilbert theory [Gilbert, G. A. (1955), *Discussions Faraday Soc. 20*, 68; Gilbert, G. A. (1963), *Ultracentrifugal Anal. Theory Expt. Conf. Rockefeller Inst. 1962*, 73; Gilbert, G. A., and Jenkins, R. C. L. (1963), *Ultracentrifugal Anal. Theory Expt. Conf. Rockefeller Inst. 1962*, 59; Nichol, L. Bethune, J. L., Kegeles, G., and Hess, E. L. (1964), *Proteins 2*, 305]. An analysis of some sedimentation equilibrium data on lysozyme at 15 and 25° and at pH 6.7 (20°) is reported. At both temperatures the lysozyme appears to undergo a monomer–dimer association; the association is more pronounced at 15°. A discussion of sources of experimental error is also included.

There are many molecules (soaps, detergents, some proteins, some chelate compounds, etc.) that undergo in solution reversible association reactions of the types

$$nP_1 \rightleftharpoons P_n, \qquad n = 2, 3, \ldots \tag{1}$$

or

$$nP_1 \rightleftharpoons qP_2 + mP_3 + \ldots \tag{2}$$

Here P represents a molecule, generally a macromolecule, undergoing the association–dissociation reaction. From sedimentation equilibrium experiments on these chemically reacting systems the quantities c (concentration) and $M_{w\,app}$ are obtained. In ideal solutions $M_{w\,app}$[1] (the apparent weight molecular weight) becomes $M_{w(c)}$ (the weight-average molecular weight).

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[1] The quantity $M_{w\,app}$ is the apparent weight-average molecular weight at any radial position, r, in the ultracentrifuge cell between the meniscus position, $r = a$, and the cell bottom position, $r = b$. $M_{w\,app}$ is given by the equation $d \ln c/d(r^2) = AM_{w\,app}$. Here, $A = (1 - \bar{v})\omega^2/2RT$; $\bar{v}$ = partial specific volume of the associating solute. (It is assumed that $\bar{v}_{Monomer} = \bar{v}_{Dimer} = \bar{v}$, *i.e.*, all partial specific volumes of the associating species are equal.) ρ = the density of the solution; ω = the angular velocity ($\omega = 2\pi$ rps); R = the gas constant; T = the absolute temperature; $c = c_r$ = the total concentration of the associating macromolecule in the solution column of the ultracentrifuge cell at any radial position between the meniscus ($r = a$) and the cell bottom ($r = b$).

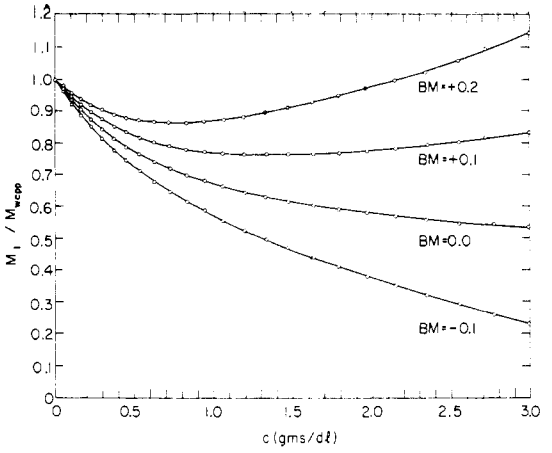

FIGURE 1: Monomer–dimer–trimer association. In this figure the effect of varying the value of the virial coefficient (BM_1) is shown for a hypothetical monomer–dimer–trimer association. In constructing the plots of $M_1/M_{w\,app}$ *vs.* c the values of K_2 (0.65) and K_3 (0.50) are held constant, while the value of BM_1 is varied from −0.10 (lowest curve) to +0.20 (uppermost curve). Note that there is a minimum in the plots of $M_1/M_{w\,app}$ *vs.* c when BM_1 is positive, and that the minimum is shifted closer to zero concentration with $BM_1 = 0.20$. There is no minimum present in these plots when BM_1 is zero or negative.

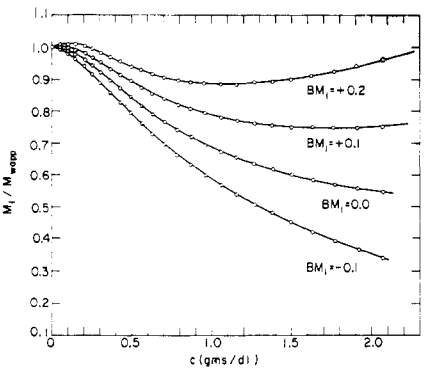

FIGURE 2: Monomer–trimer association. Here are shown plots of $M_1/M_{w\,app}$ *vs.* c for a monomer–trimer association; for these plots the value of K_3 (0.50) remains fixed, while the virial coefficient (BM_1) is varied from −0.10 to +0.20. Again one observes a minimum in the plot of $M_1/M_{w\,app}$ *vs.* c for positive values of BM_1, with the minimum being shifted closer to zero concentration for the larger positive value of BM_1. In addition, with positive values of BM_1, there is a slight maximum in the plot of $M_1/M_{w\,app}$ *vs.* c near zero concentration; this shows up better in the uppermost plot ($BM_1 = +0.20$).

Using the two quantities c and $M_{w\,app}$, it has been shown how to obtain f_a, the apparent weight fraction of monomer, and a quantity L, where $L = K_2 - BM_1$ (Adams and Williams, 1964), as well as the apparent number-average ($M_{n\,app}$[2]) molecular weight (Adams, 1965). There are two classes of unknowns, the equilibrium constant or constants (K_i) and the virial coefficient or nonideal term (BM_1), that are evaluated from these experiments. These quantities, the K_i and BM_1, are contained in the expressions for the average molecular or apparent average molecular weights, the $M_{w\,app}$ and the $M_{n\,app}$. Previously the mathematical theory for obtaining these quantities from the experimentally derived items—c, $M_{w\,app}$, $M_{n\,app}$, f_a, and L—has been set down (Adams, 1965). The purpose of this paper is to translate the theory into practice; in doing so, detailed calculations will be carried out with real and synthetic examples.

Tests with Synthetic Examples

Synthetic examples of the association equilibria described by eq 1 and 2 have been constructed; in addition to being useful for our own illumination and showing the shapes of the curves of $M_1/M_{w\,app}$ *vs.* c for the particular examples considered, they also answered the vital question as to whether one can indeed obtain the desired information, *i.e.*, K_2, BM_1, $M_{w(c)}$, $M_{n(c)}$, etc., from the experimentally available data, namely $M_{w\,app}$[1] and c, the concentration in grams per deciliter. Figure 1 shows a plot of $M_1/M_{w\,app}$ *vs.* c for a synthetic monomer–dimer–trimer system. The plots used in Figure 1 are based on the equation (Adams and Williams, 1964; Adams, 1965)

$$M_1/M_{w\,app} = M_1/M_{w(c)} + BM_1c + 0(c^2) \qquad (3)$$

Here it is assumed, as has been done previously, that the logarithm of the activity coefficient, y_i, on the c concentration scale for each associating species i can be described by

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

For the examples shown in Figure 1, the constant values of K_2 and K_3 have been used, but the value of BM_1 has been varied. One notes that if BM_1 (the second virial coefficient or nonideal term) is positive, then a plot of $M_1/M_{w\,app}$ against c shows a minimum; on the other hand, when BM_1 is zero or negative there is no minimum in this type of plot. As a consequence of eq 4 it should be noted the following equa-

[2] The quantity $M_{n\,app}$ is the apparent number-average molecular weight at any radial position in the ultracentrifuge between the meniscus and the cell bottom. For ideal solutions, $M_{n\,app}$ becomes $M_{n(c)}$. It must be reemphasized that $M_{n\,app}$ or $M_{n(c)}$ is obtained from a series of sedimentation equilibrium experiments at different initial concentrations. The values of $M_1/M_{w\,app}$ (or $M_1/M_{w(c)}$) from each experiment are plotted *vs.* c (the concentration at the radial position r corresponding to $M_{w\,app}$ or $M_{w(c)}$) and extrapolated to $c = 0$. The area under the curve of the plot of $M_1/M_{w\,app}$ (or $M_1/M_{w(c)}$) *vs.* c gives $M_{n\,app}$ or $M_{n(c)}$, as eq 6b or 6a shows.

tions obtain. The total concentration for the associating macromolecule is given by

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

for a monomer–dimer–trimer equilibrium, or

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

for a monomer–n-mer equilibrium. In addition it should be noted for these equilibria that

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

and also that

$$K_n = c_n/c_1^n, \qquad n = 2, 3, \ldots \qquad (5d)$$

One interesting feature about the positive virial coefficient and the minimum it exhibits is that for this particular case the quantity $M_1/M_{w\ app}$ does *not* have a one-to-one correspondence with c (see Figure 1), and consequently neither does the plot of $M_{w\ app}$ *vs.* c have a unique one-to-one correspondence. Thus, the statement by Adams and Fujita (1963) that $M_{w\ app}$ has a one-to-one correspondence with c is only true whenever $BM_1 = 0$ or whenever $BM_1 < 0$.

Figure 2 shows the plots of $M_1/M_{w\ app}$ *vs.* c at various values of BM_1 for a monomer–trimer equilibrium. An interesting feature of this plot is that whenever $BM_1 > 0$, the plot of $M_1/M_{w\ app}$ *vs.* c will show a maximum near but not at the origin, *i.e.*, near but not at $c = 0$. In addition with positive values of BM_1 there is also a minimum at higher values of c in the plot of $M_1/M_{w\ app}$ *vs.* c. Whenever BM_1 is negative there is neither a maximum in the plot of $M_1/M_{w\ app}$ *vs.* c near $c = 0$ nor is there a minimum further out in the plot; for $BM_1 = 0$ this type of plot will not show a minimum further out from $c = 0$, but it will have a limiting slope of zero at $c = 0$.

Previously it has been shown that the number-average molecular weight $(M_{n(c)})^2$ can be obtained for associating systems described by eq 1 and 2 by the equation (Adams, 1965)

$$\int_0^c \frac{M_1}{M_{w(c)}}\,dc = \frac{CM_1}{M_{n(c)}} \qquad (6a)$$

for ideal solutions; with nonideal solutions one gets an apparent number-average molecular weight $(M_{n\ app})^2$ from the equation

$$\int_0^c \frac{M_1}{M_{w\ app}}\,dc = \frac{CM_1}{M_{n\ app}} \qquad (6b)$$

In Figure 3 there is a plot of $M_1/M_{w\ app}$ *vs.* c as well as a plot of $M_1/M_{n\ app}$ *vs.* c for a hypothetical, nonideal monomer–dimer–trimer association, using a value of $BM_1 = 0.2$. Although the plot of $M_1/M_{w\ app}$ *vs.* c

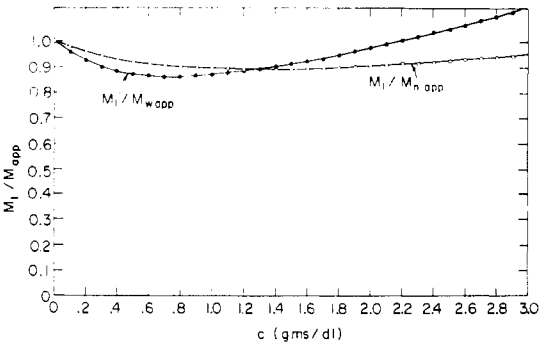

FIGURE 3: $M_1/M_{w\ app}$ and $M_1/M_{n\ app}$ *vs.* c. This figure shows a comparison of the plots of $M_1/M_{w\ app}$ (the curve starting out lower) and $M_1/M_{n\ app}$ *vs.* c for a nonideal monomer–dimer–trimer association. In constructing these plots the values of $K_2 = 0.65$, $K_3 = 0.50$, and $BM_1 = +0.20$ were used. When comparing the two plots note how the minimum is broadened and shifted away from zero concentration in the plot of $M_1/M_{n\ app}$ *vs.* c.

shows a minimum, this effect is reduced in the plot of $M_1/M_{n\ app}$ *vs.* c. It should be noted for associating systems described by eq 1 and 2 that the limiting value of $M_{w\ app}$ or $M_{n\ app}$ at $c = 0$ is M_1, *i.e.*

$$\lim_{c\to 0} M_{w\ app} = M_1 = \lim_{c\to 0} M_{n\ app} \qquad (6c)$$

In order to evaluate $cM_1/M_{n\ app}$ from eq 6b, it is necessary to plot $M_1/M_{w\ app}$ (obtained from several different sedimentation equilibrium experiments at different initial concentrations) *vs.* its corresponding c, the concentration at any radial position in the ultracentrifuge cell solution column between the meniscus and the cell bottom, as is done in Figure 3. Equation 6c tells us that

$$\lim_{c\to 0} M_1/M_{w\ app} = 1$$

The required integration can be done numerically using Simpsom's rule or the trapezoidal rule. The data used in plotting $M_1/M_{w\ app}$ *vs.* c in Figure 3 are given in Table I. The first column gives the concentration, c; the second column gives the values of $M_1/M_{w\ app}$. In the third column are reported the values of $cM_1/M_{n\ app}$, obtained in this case by the trapezoidal rule. Finally, the fourth column tabulates the values of $\alpha = c_1e^{BM_1c}$, the quantity α being calculated from f_a, the apparent weight fraction of monomer, since

$$\alpha = cf_a = c_1e^{BM_1c} \qquad (7a)$$

Adams and Williams (1964) have shown that the apparent weight fraction of monomer, f_a, can be calculated from the equation

$$\ln f_a = \int_0^c \left(\frac{M_1}{M_{w\,app}} - 1\right)\frac{dc}{c} = \ln f + BM_1c \quad (7b)$$

where $f = c_1/c$ = weight fraction of monomer. In order to evaluate $\ln f_a$ from eq 7b it is necessary to plot $(M_1/M_{w\,app} - 1)/c$ *vs.* c, noting that

$$\lim_{c\to 0}\left(\frac{M_1}{M_{w\,app}} - 1\right)\Big/c = -K_2 + BM_1$$

for a monomer–dimer–trimer [and n-mer(s)] equilibrium or BM_1 for a monomer–n-mer ($n > 3$) equilibrium; thus the quantity $\ln f_a$ is evaluated numerically from this type of plot. With the data given in columns 1 and 2 of Table I, one can construct the necessary graphs to obtain the data reported in columns 3 and 4 of Table I.

TABLE I: Values of c, $M_1/M_{w\,app}$, $cM_1/M_{n\,app}$, and $\alpha = c_1e^{BM_1c}$ for a Synthetic Monomer–Dimer–Trimer Equilibrium.[a]

c (g/dl)	$M_1/M_{w\,app}$	$cM_1/M_{n\,app}$	$c_1e^{BM_1c}$
0.00	1.000	0.000	0.000
0.10	0.959	0.098	...
0.20	0.924	0.192	...
0.30	0.900	0.283	...
0.40	0.883	0.373	0.345
0.50	0.871	0.460	0.420
0.70	0.861	0.633	0.562
0.80	0.861	0.719	0.630
1.00	0.868	0.892	0.764
1.50	0.911	1.336	1.096
2.00	0.975	1.806	1.437
2.50	1.049	2.312	1.799
3.00	1.130	2.856	2.187

[a] M_1 = 10,000; K_2 = 0.65; K_3 = 0.50; BM_1 = +0.20.

Having shown how to obtain the quantities α and $cM_1/M_{n\,app}$, we can now proceed to test for the type of association present. Let us consider a nonideal monomer–dimer–trimer equilibrium with M_1 = 10,000, K_2 = 0.65, K_3 = 0.50 at c = 3.00 g/dl. From an experimental point of view the problem is this: only c and $M_{w\,app}$ (or $M_1/M_{w\,app}$) are measured; can these data be used to (1) establish the type of equilibrium, and (2) to evaluate $M_{w(c)}$, $M_{n(c)}$, BM_1, K_2, and K_3? The answer to both questions is a definite yes! This is how it is done. The data for this association reaction are plotted in Figure 1 in the curve for BM_1 = 0.10, as well as other values of c and $M_{w\,app}$. By using these data and applying eq 7a and 7b it is found that α = 1.620, and with the help of eq 6b it is noted that $cM_1/M_{n\,app}$ = 2.406 at c = 3.00 g/dl. In addition it can be noted that the quantity $L = K_2 - BM_1$ = 0.55; this quantity is obtained from the limiting slope of a plot of $M_1/M_{w\,app}$ *vs.* c, since for the monomer–dimer or the monomer–dimer–trimer [and n-mer(s)] Adams and Williams (1964) have shown that

$$\lim_{c\to 0} d(M_1/M_{w\,app})/dc = -K_2 + BM_1 \quad (8)$$

In Table II it is shown how one tests for the various possible types of association that are thought to be present. First these possibilities are listed; this is done in column 1 of Table II. Next the equations for the various types of associations thought to be present are written down; this will be illustrated for two possibilities, the monomer–dimer and the monomer–dimer–trimer association. First let us consider the monomer–dimer association $2P_1 \rightleftarrows P_2$.

For the monomer–dimer association Adams' (1965) eq 15a and 15b can be used provided that eq 4, of this paper, applies; thus, using $M_{w\,app}$ and c, the following equations obtains

$$2c = \frac{1}{\dfrac{M_1}{cM_{w\,app}} - BM_1} - \alpha e^{-BM_1c} \quad (9a)$$

Had $cM_1/M_{n\,app}$ and c been used, the result would be

$$\frac{2cM_1}{M_{n\,app}} - c = c_1 + BM_1c^2 = \alpha e^{BM_1c} + BM_1c^2 \quad (9b)$$

The only unknown quantity in eq 9a or 9b is the quantity BM_1; thus one makes successive approximations of the unknown, BM_1, until the left-hand side of eq 9a or 9b is the same as the right-hand side. Column 2 in Table II lists the quantity to be approximated, $2c$ or $(2cM_1/M_{n\,app}) - c$ for the monomer–dimer association; the value of the approximation is listed in column 3. The experimentally observed values of the quantity which is to be approximated [$2c$ or $(2cM_1/M_{n\,app}) - c$] are listed in column 4, and the values of BM_1 used in making the approximations are given in column 5. Thus using eq 9a and BM_1 = +0.10 for the possible monomer–dimer association, at c = 3.00 g/dl it is observed that 6.00 $\neq$ 5.65 + 1.20 = 6.85. Here αe^{-BM_1c} = 1.20 and $1/[(M_1/cM_{w\,app}) - BM_1]$ = 5.65 have been used. It would appear that there might be a value of BM_1 that would give a solution to eq 9a, and it is shown in Table III how one looks for this possibility. The data in Table III make it appear that at BM_1 = 0.062 there is a solution to eq 9a; with this value of BM_1 (0.062) it is found that $c_1 = \alpha e^{-BM_1c}$ = 1.345 and that $1/[(M_1/cM_{w\,app}) - BM_1]$ = 4.658. The quantity $K_1c_1^2$ is evaluated from the relation

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

TABLE II: Test for Type of Association.[a]

Type of Assn	Quantity Calcd	Calcd Value	True Value	BM_1 Used
Monomer–dimer	$2c$	5.23	6.00	0.00
	$2c$	6.86	6.00	+0.10
	$(2cM_1/M_{n\,app}) - c$	1.62	1.81	0.00
	$(2cM_1/M_{n\,app}) - c$	2.10	1.81	+0.10
Monomer–trimer	$3c$	6.85	9.00	0.00
	$3c$	8.06	9.00	+0.10
	$(3cM_1/M_{n\,app}) - c$	3.75	4.22	+0.10
Monomer–dimer–tetramer	$4c$	11.36	12.00	0.00
	$4c$	11.13	12.00	+0.10
	$(4cM_1/M_{n\,app}) - c$	6.34	6.62	+0.10
Monomer–dimer–trimer	$(3cM_1/M_{n\,app}) - c$	4.22	4.22	+0.10
	$3c$	9.00	9.00	+0.10
	$3c$	8.30	9.00	0.00
	$(6cM_1/M_{n\,app}) - 5c$	−0.56	−0.56	+0.10
	$(6cM_1/M_{n\,app}) - 5c$	−3.26	−0.56	0.00
Monomer–dimer–trimer–tetramer[b]	$(12cM_1/M_{n\,app}) - 7c$	7.88	7.87	+0.10
		7.55	7.87	0.00

[a] The correct type of association is monomer–dimer–trimer. The true values are $M_1 = 10{,}000$, $K_2 = 0.65$, $K_3 = 0.50$, and $BM_1 = +0.10$. For these calculations the experimentally available data are $c = 3.00$ g/dl, $M_1/M_{w\,app} = 0.830$, $\alpha = c_1e^{BM_1c} = 1.620$, $cM_1/M_{n\,app} = 2.406$, $L = K_2 - BM_1 = 0.55$. [b] For this calculation $c_1 = 1.200$, $K_2c_1^2 = 0.934$, $K_3c_1^3 = 0.864$ at $BM_1 = +0.10$, and one finds $c_1 + K_2c_1^2 + K_3c_1^3 = 3.00$. Since $c = 3.00$ g/dl also, no tetramer is present and one has a monomer–dimer–trimer association present.

TABLE III: Details of the Successive Approximations for the Monomer–Dimer Association.

		$2c$ (g/dl)	
BM_1	e^{-BM_1c}	Calcd	True
0	1.000	5.23	6.00
0.05	0.8607	5.80	
0.06	0.8353	5.97	
0.062	0.8303	6.00[a]	
0.07	0.8106	6.15	
0.10	0.7408	6.86	

[a] Apparent solution; $\alpha = 1.620$, $M_1/M_{w\,app} = 0.830$, $M_1/cM_{w\,app} = 0.277$, $c = 3.00$ g/dl. Equation 9a was used for these approximations.

Applying the values given above we find that $K_2c_1^2 = 1.657$. Since $c_1 + K_2c_1^2 = 3.00_2$ and $c = 3.00$, it would appear that the analysis fails; however, this is *not* the case and one can now proceed to show that the apparent solution of eq 9a, considering a monomer–dimer association with a value of $BM_1 = 0.062$, is a false solution. In order to be a true solution, the values of BM_1, c_1, and $K_2c_1^2$ must also satisfy eq 9b or an equation based on a combination of $M_{w\,app}$ and $M_{n\,app}$. Thus, applying eq 9b, one finds at $c = 3.00$ that $1.812 \neq 0.345 + 0.558 = 1.903$, since $c_1 = 1.345$, $BM_1c^2 = 0.558$ for $BM_1 = 0.062$, and $(2cM_1/M_{n\,app}) - c = 1.812$. By combining $cM_1/M_{n\,app}$ and $1/[(M_1/cM_{w\,app}) - BM_1]$ one obtains

$$\frac{2cM_1}{M_{n\,app}} - 3c = BM_1c^2 - \frac{1}{\left(\dfrac{M_1}{cM_{w\,app}} - BM_1\right)} \qquad (9c)$$

For this example we have, for $BM_1 = 0.062$, $(2cM_1/M_{n\,app}) - c = 4.188$, $BM_1c^2 = 0.558$, and $1/[(M_1/cM_{w\,app}) - BM_1] = 4.658$; substituting these values into eq 9c we find that $4.19 \neq 4.658 - 0.558 = -4.10$. From the application of eq 9b and 9c it would appear that the monomer–dimer hypothesis might be false, although one might be accused of splitting hairs here. This difficulty can be overcome if the limiting slope of the plot of $M_1/M_{w\,app}$ *vs.* c can be used to obtain the quantity $L = K_2 - BM_1$ from eq 8; but this may be a very poor procedure since there may be a lot of uncertainty in the value of the limiting slope. When the limiting slope is used the equation for the monomer–dimer association becomes

$$c = \frac{1}{\dfrac{M_1}{cM_{w\,app}} - BM_1} - K_2c_1^2 =$$

$$\frac{1}{\frac{M_1}{cM_{w\ app}} - BM_1} - (L + BM_1)\alpha^2 e^{-2BM_1 c} \quad (9d)$$

Thus at $c = 3.00$ one would find with the previous values, remembering that $L = 0.55$, that $3.00 \neq 4.658 - 1.107 = 3.55$. Now this procedure is not recommended because of the uncertainty in evaluating the limiting slope of the plot of $M_1/M_{w\ app}$ *vs.* c; there is another and better procedure for showing that the monomer–dimer hypothesis is false. If the monomer–dimer hypothesis is correct, then the equations for the monomer–dimer–trimer association will also be satisfied, since the monomer–dimer association is a special case of the monomer–dimer–trimer association; it is the case where $K_3 = 0$. Inasmuch as the analysis does not involve any division by the K_2 term, which in this case would mean division by zero, this procedure is applicable, and the most appropriate equation to use is

$$\frac{6cM_1}{M_{n\ app}} - 5c = 2c_1 + 3BM_1c^2 - \frac{1}{\frac{M_1}{cM_{w\ app}} - BM_1} =$$

$$2\alpha e^{-BM_1 c} + 3BM_1c^2 - \frac{1}{\frac{M_1}{cM_{w\ app}} - BM_1} \quad (10a)$$

Inserting the values of $c_1 = 1.345$, $BM_1 = 0.062$, $1/[(M_1/cM_{w\ app}) - BM_1] = 4.658$, and $cM_1/M_{n\ app} = 2.406$ at $c = 3.00$ g/dl into eq 10a one finds that $-0.56 \neq 0.29$. The equation below could be tried.

$$(3cM_1/M_{n\ app}) - c = 2c_1 + (K_2c_1^2/2) + (3BM_1c^2/2) \quad (10b)$$

In this case one would obtain $4.22 \neq 4.36$. Although an equation analogous to eq 10b could be written for the apparent weight-average molecular weight ($M_{w\ app}$), it would not be useful for resolving the monomer–dimer hypothesis, since for this new equation the quantity approximated would be $3c$, and the monomer–dimer hypothesis gave answers that satisfied c and $2c$. Thus the apparent solution at $BM_1 = 0.062$ for the monomer–dimer case is definitely false. By using one or two additional equations one may be able to sort out false solutions, which one might not be able to do with only one available apparent average molecular weight such as $M_{w\ app}$. These are important points, since they show how $M_{n\ app}$ and $M_{w\ app}$ can be applied to this type of analysis. In addition one can look for inconsistencies in the false solution. The reader can prove for himself that there is also a false solution at $c = 2.00$ for the monomer–dimer hypothesis; here the necessary data are $BM_1 = 0.062$, $cM_1/M_{n\ app} = 1.607$, $M_1/M_{w\ app} = 0.8034$, $\alpha = 1.171$, and $e^{-BM_1 c} = 0.8834$. As a further aid in separating false and true solutions, one can generate the $M_1/M_{w\ app}$ *vs.* c curve to see how well the false or true solutions fit the experimental data; this type of procedure has been used by Townend and Timasheff (1960) in their light-scattering studies of the association of β-lactoglobulin. It can be shown that the false solution at $c = 3.00$ g/dl is due to an intersection between the $M_1/M_{w\ app}$ *vs.* c curves for the hypothetical monomer–dimer and the monomer–dimer–trimer association.

Similar procedures are applied to the other types of association, and it is concluded that the correct type of association is a monomer–dimer–trimer equilibrium. For this type of association the best equation to use is eq 10a as it combines $M_{w\ app}$ and $M_{n\ app}$; thus applying the data from Table II to eq 10a one obtains $-0.56 = 2.400 - 5.659 + 2.700 = -0.56$. Here we have used $c = 3.00$, $BM_1 = +0.10$, $2c_1 = 2\alpha e^{-BM_1 c} = 2.400$, $6cM_1/M_{n\ app} = 14.436$, and $1/[(M_1/cM_{w\ app}) - BM_1] = 2.700$. If one felt confident about the value of $L = K_2 - BM_1$, which is obtained from eq 8 or by other means described by Adams and Williams (1964), then eq 11d in Adams' (1965) paper could be used; this would give

$$\frac{3cM_1}{M_{n\ app}} - c = 2c_1 + \frac{K_2c_1^2}{2} + \frac{3}{2}BM_1c^2 =$$

$$2\alpha e^{-BM_1 c} + \frac{(L + BM_1)}{2}\alpha^2 e^{-2BM_1 c} + \frac{3}{2}BM_1c^2$$

Substituting the data from Table II, with $BM_1 = +0.10$, we find $4.218 = 2.400 + 0.468 + 1.350 = 4.218$. Thus the association is due to a monomer–dimer–trimer association. Equation 10a is preferred as it does not involve the use of a limiting slope from a plot of $M_1/M_{w\ app}$ (or $1/M_{w\ app}$) *vs.* c. It does require knowledge or an estimate of M_1, the monomer molecular weight. *A priori* knowledge of M_1 would be most convenient; however, M_1 could be estimated from the limiting value of a plot of $1/M_{w\ app}$ or $1/M_{n\ app}$ *vs.* c; here one should try to use a least-squares polynomial through the experimental data. Alternatively, M_1 may be estimated from a high-speed meniscus depletion experiment (Yphantis, 1964). By using eq 10a the analysis is done at *real* concentrations instead of relying on values obtained exclusively from data in the vicinity of infinite dilution of the macromolecule. An equation analogous to eq 10b could be written for the apparent weight-average molecular weight, $M_{w\ app}$ [refer to eq 13b in Adams (1965)]; the quantity approximated in this case is $3c$, and the results of this analysis are shown in Table II.

Having shown that a monomer–dimer–trimer association exists, and also knowing the values of BM_1 and c_1, how does one evaluate K_2 and K_3 as well as $M_{w(c)}$ and $M_{n(c)}$? From eq 10a or b one knows that $c_1 = 1.200$ when $c = 3.00$ and that $BM_1 = +0.10$. Using eq 13b in Adams (1965) one can get $K_2c_1^2$; thus one finds that $9.00 = 5.659 + 2.400 + K_2c_1^2$; $K_2c_1^2 = 0.941$. Here we have used $c_1 = 1.200$, $1/[(M_1/cM_{w\ app}) - BM_1] = 5.659$, and $3c = 9.00$. One can obtain the same result with eq 11 in Adams (1965). To get $K_3c_1^3$ one notes that

$$\frac{1}{\frac{M_1}{cM_{w\,app}} - BM_1} = c_1 + 2K_2c_1^2 + 3K_3c_1^3 \quad (10c)$$

Thus one has $5.659 = 1.200 + 1.882 + 3K_3c_1^3$; $K_3c_1^3 = 0.859$. In order to check these results, one substitutes them into the appropriate equation for the total concentration; thus for $c = c_1 + K_2c_1^2 + K_3c_1^3$ one finds that $3.00 = 1.20 + 0.94 + 0.86 = 3.00$. These calculations can also be done with the apparent number-average molecular weight, since

$$\frac{cM_1}{M_{n\,app}} = c_1 + \frac{K_2c_1^2}{2} + \frac{K_3c_1^3}{3} + \frac{BM_1c^2}{2} \quad (10d)$$

for the monomer–dimer–trimer equilibrium. The results of the calculations at $c = 3.00$ for the nonideal monomer–dimer–trimer association can be tabulated below in Table IV.

TABLE IV: Evaluation of K_2 and K_3 from $M_{w\,app}$ and $M_{n\,app}$ for the Hypothetical Monomer–Dimer–Trimer Association.

Item	From $M_{w\,app}$	From $M_{n\,app}$
c_1[a]	1.200	1.200
BM_1[a]	+0.10	+0.10
$K_2c_1^2$	0.941	0.936
$K_3c_1^3$	0.859	0.864
K_2	0.65	0.65
K_3	0.50	0.50

[a] The quantities c_1 and BM_1 are obtained from eq 10a which uses both $M_{n\,app}$ and $M_{w\,app}$. The total concentration is $c = 3.00$ g/dl, and the correct values for the equilibrium constants are $K_2 = 0.65$ and $K_3 = 0.50$. Equations 10c and d were used to obtain $K_3c_1^3$. Equation 10b of this paper or 13b in Adams (1965) was used to obtain $K_2c_1^2$.

One obtains the quantity $M_{n(c)}$ from the definition of $M_{n\,app}$ [see eq 9 in Adams (1965)]; thus at $c = 3.00$ one has

$$\frac{cM_1}{M_{n(c)}} = \frac{cM_1}{M_{n\,app}} - \frac{BM_1c^2}{2} =$$

$$2.406 - 0.450 = 1.956 \quad (11)$$

Since $M_1/M_{n(c)} = 0.652$ and $M_1 = 10{,}000$, one finds that $M_{n(c)} = 15{,}337$ at $c = 3.00$ g/dl. In order to obtain $M_{w(c)}$ one uses either eq 19 in Adams and Williams (1964) or eq 9a in Adams (1965); thus at $c = 3.00$ g/dl one has

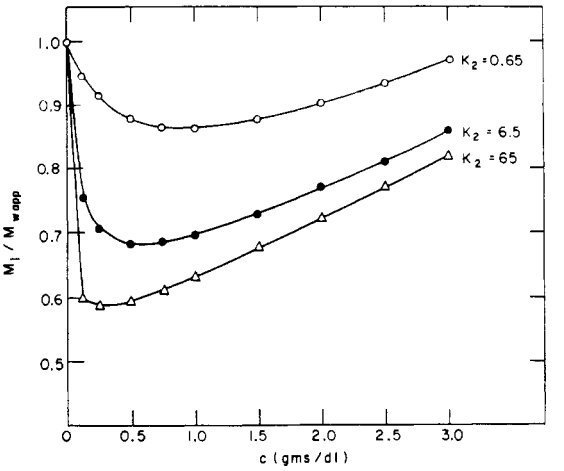

FIGURE 4: Monomer–dimer association. The plots in this figure show the effect of increasing the equilibrium constant (K_2 is varied from 0.65 to 65), while holding the virial coefficient constant ($BM_1 = 0.10$) for a nonideal monomer–dimer association. As the equilibrium constant is increased, the curves become steeper near the region where the concentration of the macromolecule tends toward zero.

$$\frac{M_1}{M_{w(c)}} = \frac{M_1}{M_{w\,app}} - BM_1c =$$

$$0.830 - 0.300 = 0.530 \quad (12)$$

Thus for $M_{w(c)}/M_1 = 1.887$ and $M_1 = 10{,}000$, one obtains $M_{w(c)} = 18.87 \times 10^3$.

In order to analyze the nonideal monomer–dimer–trimer–tetramer equilibrium, it appears at present that one needs to know the value of the quantity L. Although 30-mm cells allow one to extend the Rayleigh and the schlieren optics to lower concentrations, they may not be able to go to low enough concentrations; furthermore, there may also be adsorption of the macromolecule on the cell centerpiece as well as other disturbances. It may be possible to overcome some of these limitations with absorption optics, since this optical system can be used in many cases with very low concentrations.

Measurements of the apparent weight-average molecular weight (M_{wapp}) at very low concentrations would be quite useful in these studies, since they would be quite helpful in distinguishing monomer–n-mer associations from cases where further (and perhaps unexpected) dissociation is occurring. One recalls (see Figure 2) that there is a maximum in the vicinity of $c = 0$, but not at $c = 0$, for the plot of $M_1/M_{w\,app}$ *vs.* c for the monomer–n-mer ($n > 3$) equilibrium with a positive virial coefficient (*i.e.*, with a positive BM_1). It is possible that the trend of the data may indicate an intercept which is much lower than it should be for a monomer, whereas in reality one is dealing with a monomer–n-mer association with a positive nonideal term. Figure 4 shows the effect of different values of the equilibrium constant for a nonideal monomer–dimer equilibrium;

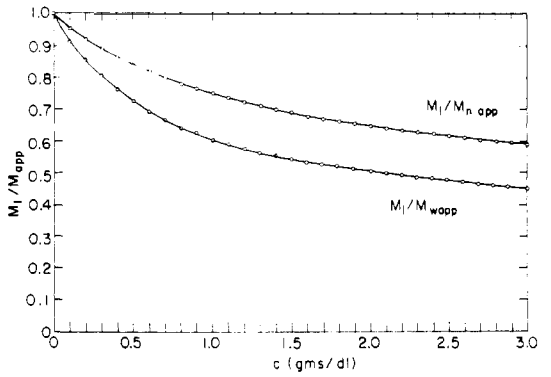

FIGURE 5: Association of α-chymotrypsin. Here are shown plots of $M_1/M_{w\ app}$ (lower curve) and $M_1/_{n\ app}$ (upper curve) *vs.* concentration (grams per deciliter) for α-chymotrypsin. These plots are based on the data of Rao and Kegeles (1958).

for all three curves the same value of the nonideal term ($BM_1 = +0.10$) has been used. It should be noted that the plots of $M_1/M_{w\ app}$ *vs.* c become quite steep in the vicinity of infinite dilution of the associating macromolecule with increasing values of K_2, the equilibrium constant, and it would appear that measurements in the very low concentration region would be necessary and useful for this case also.

It should be noted that in Table II the monomer–dimer–trimer–tetramer equilibrium appears to give a solution. Now this is *not* inconsistent if one remembers that the equations are set up (for the monomer–dimer–trimer–tetramer association) to eliminate in succession the $K_4c_1^4$ term and then the $K_3c_1^3$ term. Thus when $K_4 = 0$ one ends up with a final equation that will agree with a monomer–dimer–trimer association also. One can indeed show that for the example under consideration one is dealing with a monomer–dimer–trimer association, since the approximation will give c_1 and $K_2c_1^2$ when both sides of the equation are equal. If one originally assumes a monomer–dimer–trimer–tetramer association, using an analog of eq 5a, 6b, 8, and

$$4c = 4c_1 + 2K_2c_1^2 + 3K_3c_1^3 + 1\Big/\left(\frac{M_1}{cM_{w\ app}} - BM_1\right) \quad (13a)$$

one obtains

$$\frac{12cM_1}{M_{n\ app}} - 7c = 6c_1 + K_2c_1^2 - 6BM_1c^2 - \frac{1}{\dfrac{M_1}{cM_{w\ app}} = BM_1} = 6\alpha e^{-BM_1c} + (L + BM_1)\alpha^2e^{-2BM_1c} + 6BM_1c^2 - \frac{1}{\dfrac{M_1}{cM_{w\ app}} - BM_1} \quad (13b)$$

where $L = K_2 - BM_1$; $\alpha = c_1e^{BM_1c}$. By going through the calculations with eq 13b and subsequently using eq 13a to obtain $K_3c_1^3$, it is observed that the sum of $c_1 + K_3c_1^2 + K_3c_1^3 = 3.007$, which is the same as $c = 3.00$ g/dl, and thus it is concluded no tetramer is present.

Although the details of the analysis have been given at one concentration, this analysis could and should be done at several concentrations. In order to minimize the effects of adsorption or other sources of error, concentrations greater than 1 g/dl should be employed. The analysis described here can be extended to other equilibria, such as the monomer–*n*-mer, and the analysis can also differentiate between negative values of BM_1 and the presence of higher associating species.

Recently Elias (1965) has published a theoretical paper on monomer–dimer associations. Although he purports to have a method for evaluating the equilibrium constant and the nonideal term, it should be noted that his eq 37 implies that $1/M_{n\ app}$ becomes infinite at zero concentration of the macromolecule, and for ideal systems his eq 38 will not reduce to the appropriate equation (the equation for the case where $n = 2$) for the monomer–*n*-mer equilibrium developed by Rao and Kegeles (1958).

The Association of α-Chymotrypsin

Inasmuch as there is no flow through the air–solution meniscus or the cell bottom in an ultracentrifuge cell, Archibald (1947) pointed out that the conditions for sedimentation equilibrium existed at these boundary positions of the solution column at all times during an ultracentrifuge run. Klainer and Kegeles (1955) showed how this observation could be put into practice; this method was extended by Kegeles and Rao (1958) to chemically reacting systems. The Archibald method has been used by Rao and Kegeles (1958) to study the association of α-chymotrypsin. In their paper recourse was made to additional sedimentation velocity experiments and the subsequent application of the Gilbert theory (Gilbert, 1955, 1963; Gilbert and Jenkins, 1963; Nichol *et al.*, 1964) to help determine the type of association present. We propose to show that this analysis can be done entirely with the data available from Archibald method runs, *i.e.*, from $M_{w\ app}$ and c. Figure 5 shows a plot of $M_1/M_{w\ app}$ *vs.* c (lower curve) as well as $M_1/M_{n\ app}$ *vs.* c (upper curve); these curves have been calculated from Figure 1 of their paper (Rao and Kegeles, 1958). The analysis previously described in this paper and elsewhere (Adams, 1965) can be applied to this data. For convenience some of the data from Rao and Kegeles' (1958) work is tabulated below[3] in Table V.

[3] Since one is dealing with a multicomponent system containing an associating macromolecule which may ionize, one should follow previous conventions (Casassa and Eisenberg, 1964; Adams, 1965) and use $M_{w\ app}{}^*$, $B^*M_1{}^*$, etc., in this analysis and discussion. For convenience the asterisks have been omitted, but the reader should be aware that in general one is analyzing the association of a macromolecular component defined by the Casassa and Eisenberg (1964) or by the Scatchard and Bregman (1959) conventions.

TABLE V: α-Chymotrypsin Data of Rao and Kegeles (1958).

c (g/dl)	$\frac{M_1}{M_{w\ app}}$	$\frac{cM_1}{M_{n\ app}}$	$\alpha(c_1e^{BM_1c})$
0.8	0.642	0.625	0.481
1.0	0.603	0.749	0.552
1.5	0.541	1.034	0.696
2.0	0.504	1.295	0.809
2.5	0.476	1.540	0.902
3.0	0.450	1.771	0.982

One built-in disadvantage of the Archibald method is that in order to evaluate $M_{w\ app}$ one has to extrapolate the concentration gradient (dc/dr) data to the meniscus and/or the cell bottom. This extrapolation involves taking data from a continuous region to a discontinuous region (the meniscus or the cell bottom), and this extrapolation may be affected by optical distortion in the vicinity of these discontinuous regions.

An analysis of the data of Rao and Kegeles (1958) leads to two possibilities for the type of association: monomer–dimer–trimer–tetramer or monomer–dimer–trimer equilibria. In order to consider the monomer–dimer–trimer–tetramer association at present one has to use the limiting slope from a plot of $M_1/M_{w\ app}$ *vs.* c to obtain the quantity $L = K_2 - BM_1$ or use the intercept in a plot of $[(M_1/M_{w\ app}) - 1]/c$. From the limiting slope of a plot of $M_1/M_{w\ app}$ *vs.* c one obtains $L = 1.06_6$, whereas the intercept from a plot of $[(M_1/M_{w\ app}) - 1]/c$ *vs.* c gives $L = 1.07_6$; the average from the two methods is $L = 1.07_1$. In obtaining the quantity L a finite difference technique (Scarborough, 1962) was employed, namely Newton's method for forward interpolation. In considering the monomer–dimer–trimer–tetramer equilibrium, it appears that one must use eq 13b at present. Applying the analysis to the monomer–dimer–trimer–tetramer case one obtains these average values: $K_2 = 1.078$, $BM_1 = 0.00_4$, $K_3 = 0.27$, and $K_4 = 0.88$, which appears to indicate an ideal solution for this case. The main stumbling block in this analysis is the value of the limiting slope in the plot of $M_1/M_{w\ app}$ *vs.* c or in the intercept of the plot of $[(M_1/M_{w\ app}) - 1]/c$ *vs.* c. Unfortunately, the data in this region have to be obtained from very dilute solutions where errors may be fairly large; Rao and Kegeles (1958) were quite aware of these difficulties in obtaining data in this region. To overcome some of these difficulties, they used 30-mm centerpieces in their ultracentrifuge cells, which increases the optical magnification by a factor of 2.5. For the value of $BM_1 = 0.004$ the uncertainty is ± 0.003; thus one can assume in eq 13b that $BM_1 = 0$ and that $\alpha = c_1$. Applying these assumptions to the α-chymotrypsin data of Rao and Kegeles (1958) one can solve eq 13b for $K_2c_1^2$ and then obtain K_2. Doing this one obtains $K_2 = 1.06$ at $c = 1.00$ g/dl and $K_2 = 1.03$ at $c = 1.50$ g/dl. Although this technique avoids using the quantity L, it is nevertheless based on a first estimate which uses L and is only applicable to ideal solutions where $BM_1 = 0$.

When one considers the possibility o a monomer–dimer–trimer equilibrium, one need *not* use the limiting slope of a plot of $M_1/M_{w\ app}$ *vs.* c in evaluating the results. Thus for this system one uses eq 10a. The results of the analysis using this equation are summarized in Table VI. We have not shown the tests for the other types of associations which may be present, such as monomer–dimer or monomer–trimer; although these possibilities were considered, our results gave us confidence that these other possibilities may be eliminated. It should be noted that the plot of $M_1/M_{w\ app}$ does not indicate a minimum; therefore, BM_1 is not likely to be positive, unless it be very close to zero.

TABLE VI: α-Chymotrypsin Data of Rao and Kegeles and Details of the Successive Approximations for the Monomer–Dimer–Trimer Association.

c (g/dl)	BM_1	$(6cM_1/M_{n\ app}) - 5c$ Calcd	Obsd
3.00	−0.018	−4.37	−4.37
2.50	−0.018	−3.25	−3.26
2.00	−0.018	−2.24	−2.23
1.50	−0.018	−1.33	−1.30

c	c_1	K_2	K_3	Σc_i*
3.00	1.037	0.90	0.93	3.02
2.50	0.943	0.91	0.89	2.51
2.00	0.839	0.89	0.91	2.01
1.50	0.715	0.84	0.98	1.51
	Av	0.89	0.92	

$\Sigma c_i^* = c_1 + \bar{K}_2c_1^2 + \bar{K}_3c_1^3$; $\bar{K}_i$ = average value of the equilibrium constant.

Check on the above analysis by insertion of the values obtained from the monomer–dimer–trimer analysis into eq 13b for a monomer–dimer–trimer–tetramer equilibrium:

c	$(12cM_1/M_{n\ app}) - 7c$ Calcd	Obsd	BM_1 Used
3.0	0.26	0.25	−0.018
2.5	0.96	0.98	−0.018
2.0	1.51	1.54	−0.018
1.5	1.86	1.91	−0.018

Considering the monomer–dimer–trimer possibility, it is interesting to note that the calculated value of L ($=K_2 - BM_1$) is 0.91. When Rao and Kegeles (1958) assumed a monomer–dimer–trimer equilibrium they obtained $K_{2_{dissocn}}$ (the dimer dissociation equilibrium constant) = 11.1, using the grams-per-liter con-

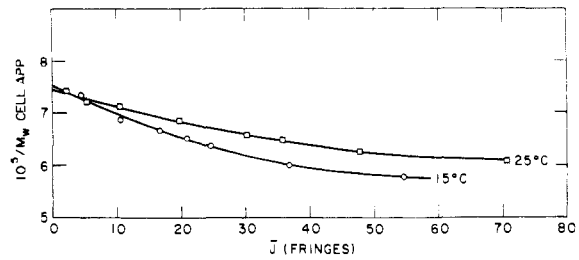

FIGURE 6: Lysozyme at 15 and 25°. The plots of $1/M_{w\ cell\ app}$ *vs.* $\bar{J}$ for lysozyme at 15 and 25° are displayed together for comparison. The curvature in these plots suggests association, and it is apparent that there is more association at 15°. It should be emphasized that these plots can be used only for display purposes with associating systems, as there is no theory presently available for using $M_{w\ cell\ app}$ to evaluate equilibrium constants and nonideal terms.

centration scale. On the grams-per-deciliter concentration $K_{2_{dissocn}} = 1.11$; the reciprocal of this quantity is the dimerization association equilibrium constant which for this case is $K_{2_{assocn}} = 1/1.11 = 0.90$. This value for $K_{2_{assocn}}$ (0.90) agrees remarkably well with our value of $K_{2_{assocn}} = 0.89$. One should note that the value of $K_{2_{assocn}}$ calculated from the data of Rao and Kegeles (1958) is really $K_{2_{assocn}} - BM_1$; thus their value of $K_{2_{assocn}} - BM_1$ of 0.90 and our calculated value of 0.91 are essentially the same. Rao and Kegeles obtained their value of $K_{2_{assocn}}$ (0.90) by a least-squares treatment of their data. On the grams-per-deciliter concentration scale their value of the association equilibrium constant for the trimer is $K_{3_{assocn}} = 1.16$, whereas our value for $K_{3_{assocn}}$ is 0.92.

By performing sedimentation equilibrium experiments on chemically reacting systems, the need for doing sedimentation velocity experiments and subsequently applying the data from these experiments to the Gilbert theory (Gilbert, 1955, 1963; Gilbert and Jenkins, 1963; Nichol *et al.*, 1964) to test for the type of association is avoided. Our analysis and the analysis of Rao and Kegeles (1958) indicate the possibility of tetramer being present. To show if this was true it was attempted to calculate $M_{z\ app}$; for sedimentation equilibrium experiments on chemically reacting systems described by eq 1 and 2 it is noted that $M_{zr} = M_{z(c)}$. Wales (1948) has given equations for calculating M_{zr} in nonreacting systems, and these equations have been extended to chemically reacting systems (Adams, 1964). For ideal systems, the equation for $M_{z(c)}$ is

$$M_{z(c)} = \mathrm{d}(cM_{w(c)})/\mathrm{d}c \qquad (14a)$$

With nonideal systems eq 14a gives $M_{z\ app}$, where

$$M_{z\ app} = \mathrm{d}(cM_{w\ app})/\mathrm{d}c = M_{z(c)}/(1 + BcM_{w(c)})^2 \qquad (14b)$$

Unfortunately the use of $M_{z\ app}$ by itself did not resolve the issue, and the question will remain unresolved until other relations are developed. When the data for either possible association of the α-chymotrypsin is used to generate the curve of $M_1/M_{w\ app}$ *vs.* c, both cases appeared to agree reasonably well with the observed curve of $M_1/M_{w\ app}$ *vs.* c. Attempts by us to do sedimentation equilibrium experiments on α-chymotrypsin have been abandoned because of apparent heterogeneity on polyacrylamide gel electrophoresis[4] (even "chromatographically homogeneous" material appeared heterogeneous) and possible autolysis.

The Association of Lysozyme at pH 6.7

According to Sophianopoulos and Van Holde (1964), lysozyme is supposed to undergo a monomer–dimer association in the region of pH 5–9, and at higher pH values higher associating species may be present. It seemed to us that lysozyme would be a good material to use in verifying the method developed by Adams (1965) for analyzing chemically reacting systems. The analytical methods used by Sophianopoulos and Van Holde (1964) were based on the assumption that the solution was ideal; this restriction is unnecessary in the method developed by Adams (1965).

For the experiments reported here the conditions chosen were such that a monomer–dimer association should be present. Commercially available hen egg lysozyme (Worthington), as well as some lysozyme prepared by Professor A. J. Sophianopoulos,[5] were used in these experiments. Fractionation of the commercial preparation with ammonium sulfate (Sophianopoulos *et al.*, 1962) was carried out, but very little if any impurity was noted. The composition of the buffer was 0.15 M NaCl and 0.005 M each of NaH_2PO_4 and Na_2HPO_4; at 20° the pH of the buffer was $6.7_0 \pm 0.05$. All protein solutions were dialyzed against the buffer in the cold (3–6°) for at least 24 hr before use. Initial protein solution concentrations were determined on a Brice-Phoenix differential refractometer at 5462 A. Sedimentation equilibrium experiments were carried out in a Spinco Model E analytical ultracentrifuge equipped for Rayleigh and schlieren optics; the rotor temperature was controlled by the heater and refrigeration unit built into the ultracentrifuge. Rotor temperature measurements were based on resistance (RTIC) readings of a thermistor embedded in the rotor. Short columns (*ca.* 0.23 cm) were used to achieve sedimentation equilibrium more rapidly (Svedberg and Pedersen, 1940; Van Holde and Baldwin, 1958). The experiments were carried out at 15 and 25°. Rotor speeds varied from 23,150 for the lowest initial protein concentration to 13,410 rpm for the highest initial protein concentration.

For the 25° experiments the lysozyme prepared by Professor Sophianopoulos was used. At this temperature the lysozyme appeared to associate slightly, but there

[4] We wish to thank Miss Elizabeth Stanton for doing the zone electrophoresis of the α-chymotrypsin.

[5] We are very grateful to Professor A. J. Sophianopoulos for giving us some of his lysozyme.

appeared to be a more pronounced association at 15°. Figure 6 displays a comparison of the 15 and the 25° lysozyme data. In this figure the values of $1/M_{w\ cell\ app}$ are plotted *vs.* $\bar{J}$; the quantity $\bar{J}$ is the average of the sum of the meniscus and the cell bottom fringe numbers,[6] *i.e.*, $\bar{J} = {}^1/_2(J_a + J_b)$. The plots given in Figure 6 are for display purposes only; there is no quantitative theory available at present which allows one to calculate the nonideal term and the equilibrium constant or constants from a plot of $1/M_{w\ cell\ app}$[7] *vs.* $\bar{J}$, since the relation between these two quantities is not known for chemically reacting systems. Previously it has been shown for ideal chemically reacting systems that $M_{w\ cell}$ does not represent the weight-average molecular weight at the initial protein concentration of the associating solute (Adams, 1964). It is of interest to compare the association of the lysozyme at the two different temperatures, and the data for these experiments will be reported here. In passing it is of interest to note that Halwer *et al.* (1951) have observed a monodisperse behavior of lysozyme in 0.1 M NaCl at 25° and pH 6.2; their light-scattering plots of $1/M_{w\ app}$ *vs.* c were linear with a negative slope. Similar results were observed by Halwer *et al.* (1951) with lactoglobulin, but in this case the slopes were smaller.

In order to convert from fringes to concentrations in grams per deciliter, it has been assumed that the value of dn/dc (converted to dl/g) reported by Halwer *et al.* (1951) applies under our experimental conditions. There is some support for this assumption as Bruzzesi *et al.* (1965) report a similar value for dn/dc of lysozyme at pH 6.8. The differential refractometer measurements were usually done at 25°; for the 15° experiments the fringe values for the initial concentrations were calculated by assuming the following relation

$$J_0(15°) = J_0(25°)\rho H_2O(15°)/\rho H_2O(25°)$$

Likewise, it was assumed that the density of the buffer (ρ_0) at 15° was given by

$$\rho_0(15°) = \rho_0(25°)\rho H_2O(15°)/\rho H_2O(25°)$$

Figure 6 suggested that the association at 25° was slight; accordingly a quadratic polynomial was fitted through the $M_{w\ app}$ *vs.* J data.[8] Table VII displays the values of J, $M_1/M_{w\ app}$, c, and α used in this analysis; the values of α, $M_1/M_{w\ app}$, and $cM_1/M_{n\ app}$ were obtained from the quadratic polynomial. As a starter only the monomer–dimer association was assumed to be present; the results of this analysis are displayed in Table VIII. For the monomer–dimer analysis one uses eq 9c; both sides of the equation are equal when one chooses the correct value of BM_1. The quantity c_1 was calculated from the relation $(2cM_1/M_{n\ app}) - c = c_1 + BM_1c^2$ or from the equation

$$2c = c_1 + \frac{1}{\dfrac{M_1}{cM_{w\ app}} - BM_1} \qquad (15a)$$

The quantity c_2 (*i.e.*, $K_2c_1^2$) was calculated from

$$\frac{2cM_1}{M_{n\ app}} = 2c_1 + K_2c_1^2 + BM_1c^2 \qquad (15b)$$

or alternatively from

$$1\Big/\left(\frac{M_1}{cM_{w\ app}} - BM_1\right) = c_1 + 2K_2c_1^2 \qquad (15c)$$

If a monomer–dimer association is correct the results will also satisfy the equations for a monomer–dimer–trimer association also; for this test in Table VIII eq 10a has been used. The trend of the $M_{w\ app}$ data indicate no maximum up to 85 fringes (over 2 g/dl), nor does the trend of the $M_1/M_{w\ app}$ data displayed in Table VII indicate a minimum before 85 fringes, so that it appears that BM_1 is very small if it be positive. A large negative value of BM_1 would make it appear that more associa-

TABLE VII: Lysozyme Data at 25°.[a]

J (fringes)	c (g/dl)	$\frac{M_1}{M_{w\ app}}$	$\frac{cM_1}{M_{n\ app}}$	α
85	2.053	0.812	1.793	1.537
83	2.005	0.813	1.754	1.496
80	1.932	0.814	1.695	1.458
78	1.884	0.815	1.656	1.428
75	1.812	0.816	1.596	1.383
73	1.763	0.818	1.557	1.355
70	1.691	0.820	1.498	1.307
65	1.570	0.824	1.398	1.230

[a] The values of dn/dc, $\bar{v}$, and ρ_0 used in these calculations are listed in Table X.

[6] The fringe or fringe number is proportional to the refractive index by the equation $J = h(n - n_0)/\lambda$. Here, J is the fringe number; h is the cell thickness (generally 12 mm); λ is the wavelength of light (5462 A in these experiments); $n - n_0$ is the difference in refractive index between the solution and the solvent (buffer). In order to convert from fringes to concentrations one notes that $n - n_0 = (dn/dc)c$, where c is the concentration and dn/dc is the refractive index increment. Although some experiments were carried out in 30-mm cells, the data in these experiments have been converted to a 12-mm basis, and all fringes reported here are based on 12-mm cell thicknesses.

[7] The quantity $M_{w\ cell\ app}$ is given by the equation $(c_b - c_a)/c_0A(b^2 - a^2) = M_{w\ cell\ app}$. Here c_0 is the original concentration of the macromolecule in the solution; the other quantities are defined in footnote 1. For ideal solutions $M_{w\ cell\ app}$ becomes $M_{w\ cell}$.

[8] For the experiments at 25° the quadratic polynomial was of the form $M_{w\ app} = A_0 + A_1J + A_2J^2$. The values of the coefficients were $A_0 = 1.3252 \times 10^4$, $A_1 = 67.521$, and $A_2 = -0.36996$.

TABLE VIII: Analysis of the Lysozyme Data at 25° as a Monomer–Dimer Association.

J (fringes)	$(2cM_1/M_{n\,app}) - 3c$ Calcd	Obsd	BM_1[c]	K_2 (dl/g)
85	−2.58	−2.57	0.02	0.29
83	−2.51	−2.51	0.02	0.29
80	−2.42	−2.41	0.02	0.29
78	−2.35	−2.34	0.02	0.29
75	−2.24	−2.24	0.01	0.26
73	−2.18	−2.17	0.01	0.25
70	−2.08	−2.08	0.01	0.25
65	−1.91	−1.92	0.01	0.26
80	−2.375	−2.406	0	
	−2.396		0.01	
	−2.419		0.02	
	−2.508		0.05	
73	−2.155	−2.175	0	
	−2.172		0.01	
	−2.190		0.02	

$\bar{K}_2 = 0.27 \pm 0.02$, $\overline{BM_1} = 0.02 \pm 0.01$.

Check on the calculations by substitution of the values obtained for the monomer–dimer association into the eq 10a for a monomer–dimer–trimer association:

J (fringes)	$(6cM_1/M_{n\,app}) - 5c$ Obsd	Calcd	
85	0.49	0.54[a]	0.49[b]
83	0.50	0.52	0.49
80	0.51	0.54	0.49
78	0.52	0.54	0.50
75	0.52	0.56	0.52
73	0.53	0.55	0.53
70	0.53	0.55	0.54

[a] These values are obtained by using $c_1 = \alpha e^{-BM_1 c}$ in eq 10a. [b] These values are obtained by using $\bar{c}_1$ in eq 10a; $\bar{c}_1$ is the average of the values of c_1 calculated from eq 9a and 9b.

tion was occurring (see Figure 1) and this also is not evident. Since the monomer–dimer–trimer equation is satisfied by the values for a monomer–dimer association, it appears unlikely that any monomer–dimer–trimer association is present. The other possibility to consider is a monomer–trimer association; here the equation to use is

$$(3cM_1/M_{n\,app}) - 4c = \frac{3BM_1c^2}{2} - \frac{1}{\frac{M_1}{cM_{w\,app}} - BM_1} \quad (15d)$$

TABLE IX: Analysis of Lysozyme at 25° as a Monomer–Trimer Association.

J (fringes)	$(3cM_1/M_{n\,app}) - 4c$ Calcd	Obsd	BM_1
85	−2.86	−2.83	0.12
83	−2.77	−2.76	0.12
80	−2.65	−2.64	0.12
78	−2.56	−2.57	0.12

Check on the calculations by substitution of the results of the monomer–trimer association into eq 10a for a monomer–dimer–trimer association:

J (fringes)	$(6cM_1/M_{n\,app}) - 5c$ Calcd	Obsd	$2\alpha e^{-BM_1c}$
85	0.30	0.49	2.403
83	0.30	0.50	2.352
80	0.37	0.51	2.348
78	0.36	0.56	2.278

The results of this analysis are shown in Table IX. If a monomer–trimer association were present one would obtain $BM_1 = 0.12$; this value of BM_1 would lead one to expect a maximum in the $M_{w\,app}$ *vs.* c data (or a minimum in the $M_1/M_{w\,app}$ *vs.* c data). Such a maximum is not observed at concentrations up to 85 fringes. Furthermore, one can consider that the monomer–trimer association is a special case of the monomer–dimer–trimer association, the case where $K_2 = 0$. Thus if the monomer–trimer association hypothesis is correct, then the data for this case must also satisfy eq 10a for the monomer–dimer–trimer association. Since the calculations in Table IX indicate that the monomer–trimer hypothesis does not satisfy eq 10a and since no maximum in the $M_{w\,app}$ at various c data (or a minimum in the $M_1/M_{w\,app}$ at various c data) is observed, one concludes that no monomer–trimer association is present.

For the lysozyme data at 15° a quadratic polynomial[9] was fitted through the $1/M_{w\,app}$ *vs.* J (fringe) data. The values of $M_1/M_{w\,app}$, J, c, $cM_1/M_{n\,app}$, and α used in the analysis are given in Table X and the results of the analysis of the lysozyme data at 15°, considering a monomer–dimer association to be present, are shown in Table XI. It is apparent from the data here and also from Figure 6 that the association is more pronounced at the lower temperature. A similar increase of association with decreasing temperature has been observed by Townend and Timasheff (1960) and also by Kumosinski and Timasheff (1965) on the association of β-lactoglobulin.

Some differences in the values of c_1 calculated from

[9] The polynomial used with the 15° lysozyme experiments was of the form $10^5/M_{w\,app} = A_0 + A_1J + A_2J^2$. The values of the coefficients were $A_0 = 7.684$, $A_1 = -6.941 \times 10^{-2}$, and $A_2 = 5.616 \times 10^{-4}$.

TABLE X: Lysozyme Data at 15°.[a]

J (fringes)	c (g/dl)	$\frac{M_1}{M_{w\ app}}$	$\frac{cM_1}{M_{n\ app}}$	α
60	1.449	0.724	1.186	0.964
58	1.401	0.725	1.151	0.941
56	1.353	0.726	1.116	
55	1.329	0.727	1.098	0.905
54	1.304	0.728	1.081	
52	1.256	0.732	1.046	
50	1.208	0.734	1.010	0.845
48	1.159	0.737	0.975	
45	1.087	0.744	0.921	

[a] $dn/dc = 1.888 \times 10^{-3}$ dl/g at 25° and 5462 A [this value of the refractive index increment is taken from the data of Halwer *et al.* (1951), and it is assumed to be the same for the experimental conditions reported here]; $\bar{v} = 0.703$ at 15° [this value of $\bar{v}$ is based on the value reported by Sophianopoulos *et al.* (1962); tables in the appendices of Svedberg and Pedersen's (1940) monograph were used in interpolating the $\bar{v}$ to 15°]; $\bar{v} = 0.705$ at 25°; $\rho_0 = 1.0054$ at 25°. Buffer: 0.15 M NaCl and 0.005 M each Na_2HPO_4 and NaH_2PO_4, pH 6.7 at 20°.

TABLE XI: Analysis of the Lysozyme Data at 15° as a Monomer–Dimer Association.

J (fringes)	$(2cM_1/M_{n\ app}) - 3c$ Calcd	Obsd	K_2 (dl/g)	BM_1
60	−1.97	−1.98	0.52	−0.02
58	−1.90	−1.90	0.52	−0.02
56	−1.83	−1.83	0.52	−0.02
55	−1.80	−1.79	0.52	−0.02
54	−1.75	−1.75	0.48	−0.03
52	−1.68	−1.68	0.48	−0.03
50	−1.61	−1.60	0.48	−0.03
48	−1.53	−1.53	0.44	−0.04
45	−1.43	−1.42	0.44	−0.04

The equation used in these approximations is $(2cM_1/M_{n\ app}) - 3c = BM_1c^2 - 1/[(M_1/cM_{w\ app}) - BM_1]$; $\bar{K}_2 = 0.49 \pm 0.03$; $\bar{BM}_1 = -0.03 \pm 0.01$.

Check on the analysis by substitution of the values obtained above into the equation for a monomer–dimer–trimer association:

J (fringes)	$(6cM_1/M_{n\ app}) - 3c$ Calcd	Obsd	c (g/dl)
60	−0.12	−0.13 (−0.07)[a]	1.449
58	−0.11	−0.10	1.401
56	−0.07	−0.07	1.353
55	−0.06	−0.06 (−0.02)[a]	1.329
50	+0.01	+0.02	1.208
45	+0.08	+0.09	1.087

[a] Obtained by using $c_1 = \alpha e^{-BM_1c}$.

eq 9c, 15a, or 7a were noted at 25 and 15°. At 15° for example one obtains for $J = 60$ fringes ($c = 1.449$ g/dl) and $BM_1 = -0.02$ the following values of c_1: 0.965 from eq 9c, 0.975 from eq 15a, and 0.993 from eq 7a. These differences are probably due to experimental error, and could be produced by adsorption, precession of the ultracentrifuge drive, or from other sources of error. In general one has to extrapolate the $M_1/M_{w\ app}$ data *vs.* c to zero concentration of the protein in order to analyze these systems. Adsorption, particularly if it be serious, could affect the shape of the curve so obtained, particularly in the low concentration region (0.3 g/dl and lower). This is one reason why one should carry out these experiments at high initial protein concentrations (at least 1 g/dl and higher) in addition to doing experiments at lower initial protein concentrations. It should be noted that LaBar (1965) has recently published a paper which may allow one to overcome the problems of adsorption; we have not yet tried this procedure. Errors in the optical constants can also produce erroneous values in the apparent molecular weights.

Although the data in Figure 6 look quite good, Figure 7 shows that there is quite a bit of scatter in the $M_{w\ app}$ or $1/M_{w\ app}$ *vs.* c plots; this scatter did worry us a lot. Some of the scatter may arise from mechanical feedback from the drive or from instantaneous speed fluctuations; these effects may be overcome with the new drive for the electronic speed control. Thermal gradients in the rotor may also be a source of scatter in the $M_{w\ app}$ data; it may be possible to overcome this problem by using clear rotors and also by adjusting the refrigeration unit so that the temperature of the refrigeration jacket is close (within 5° or less, if possible) to the desired operating temperature.

Another potential source of error in this method of analysis may be the salt redistribution effect; it has been assumed in the theoretical treatment (Adams and Fujita, 1963; Adams and Williams, 1964; Adams, 1965) that these effects are negligible. It should be noted that the limiting molecular weight for lysozyme was 13.4×10^3 at 25° and 13.3×10^3 at 15° from the data in Figure 6, whereas it was 13.01×10^3 at 15° using the quadratic polynomial data. A difference between the $M_{w\ app}$ (the quadratic polynomial data) and the $M_{w\ cell\ app}$ data is not surprising since the $M_{w\ app}$ are measured to lower concentrations and will influence the shape of the curve more. The low value of the molecular weight of the monomer may be due to charge effects, salt redistribution, adsorption, or to the wrong value for the partial specific volume. Similar discrepancies have been noted by K. E. Van Holde (personal communication). It has been assumed in this work that the reported partial specific volume (Sophianopoulos *et al.*, 1962) is the same as the $\bar{v}^*$ described by Casassa and Eisenberg (1964),

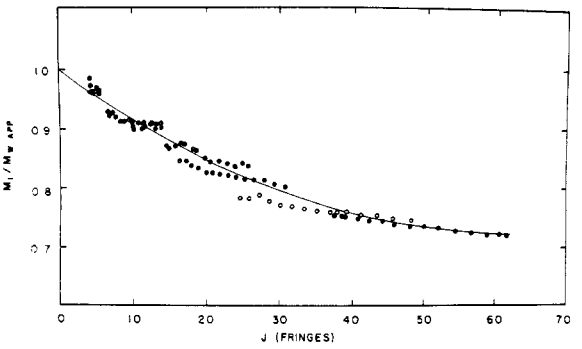

FIGURE 7: Lysozyme at 15°. The plot of $M_1/M_{w\,app}$ *vs.* J, based on a quadratic polynomial fitted through the data, is displayed here; this curve is characteristic of some associating systems (see Figure 1).

and it has also been assumed that the reported values for dn/dc (Halwer *et al.*, 1951; Bruzzesi *et al.*, 1965) are the same as dn/dc*. In sedimentation equilibrium experiments the value of the molecular weight obtained does depend on the choice of $\bar{v}$ and its reliability. Surprisingly one does not need to know $\bar{v}$ in order to evaluate BM_1, the nonideal term, or K_i, the equilibrium constant or constants. For incompressible systems (aqueous systems are generally quite incompressible), one notes that (Van Holde and Baldwin, 1958; Casassa and Eisenberg, 1964)

$$(1 - \bar{v}) = (1 - \bar{v}\rho_0)(1 - \bar{v}c/100) \qquad (16a)$$

The basic equation for sedimentation equilibrium is

$$\text{d ln } c/\text{d}(r^2) = AM_{w\,app} = AM_{w(c)}/(1 + BM_{w(c)}c) \qquad (16b)$$

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

Using eq 16a one notes that $A = A_0(1 - \bar{v}c/100)$, and substituting this relation into eq 16b one obtains

$$\frac{M_1}{M_{w\,app'}} = \frac{M_1}{M_{w(c)}} + M_1\left[B + \frac{\bar{v}}{100M_{w(c)}}\right]c = \frac{M_1}{M_{w(c)}} + B'M_1c + \ldots \qquad (16c)$$

Here, $B' = B + \bar{v}/100M_1$; $A_0M_{w\,app'} = \text{d ln } c/\text{d}(r^2)$. The error involved in replacing $\bar{v}/100M_{w(c)}$ by $v/100M_1$ is probably within or less than the experimental error. It should be noted that one can also modify eq 6a or 6b to give $M_{n(c)'}$ or $M_{n\,app'}$.

Recently some light-scattering studies havd been reported on lysozyme (Bruzzesi *et al.*, 1965) at pH 6.8 in 0.3 M potassium phosphate buffer at 20°. Their data indicated species higher than dimer may be present, which might be expected at the higher ionic strengths used by them; attempts by them to correct for nonideality were unsuccessful. It should be noted that Steiner (1953) has stated that the association reaction (in a rapid dynamic equilibria) may have a coupling effect on the fluctuations of the dipoles; this coupling effect could produce too high an observed molecular weight. The validity of this assertion is a moot point at present, and if there is no coupling effect, one should be able to apply the analyses described here to light-scattering experiments.

From the studies reported here, it does appear that one can analyze some chemically reacting systems of interest. It is hoped that these reports will encourage others to investigate these systems and to develop improved methods for analyzing them.

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References

Adams, E. T., Jr. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 509.

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

Adams, E. T., Jr., and Fujita, H. (1963), in Ultracentrifugal Analysis in Theory and Experiment, Willams, J. W., Ed., New York, N. Y., Academic, p 119.

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

Archibald, W. J. (1947), *J. Phys. Colloid Chem. 51*, 1204.

Bruzzesi, M. R., Chiancone, R., and Antonini, E. (1965), *Biochemistry 4*, 1796.

Casassa, E. F., and Eisenberg, H. (1964), *Advan. Protein Chem. 19*, 287.

Elias, H.-G. (1965), *Helv. Chim. Acta 48*, 1018.

Gilbert, G. A. (1955), *Discussions Faraday Soc. 20*, 68.

Gilbert, G. A. (1963), in Ultracentrifugal Analysis in Theory and Experiment, Williams, J. W., Ed., New York, N. Y. Academic, p 73.

Gilbert, G. A., and Jenkins, R. C. L. (1963), in Ultracentrifugal Analysis in Theory and Experiment, Williams, J. W., Ed., New York, N. Y. Academic, p 59.

Halwer, M., Nutting, G. C., and Brice, B. A. (1951), *J. Am. Chem. Soc. 73*, 2786.

Kegeles, G., and Rao, M. S. N. (1958), *J. Am. Chem. Soc. 80*, 5721.

Klainer, S. M., and Kegeles, G. (1955), *J. Phys. Chem. 59*, 952.

Kumosinski, T. F., and Timasheff, S. N. (1965), Abstracts, 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 12–17, p 76C, No. 157.

LaBar, F. E. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 31.

Nichol, L., Bethune, J. L., Kegeles, G., and Hess, E. L. (1964), *Proteins 2*, 305.

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 56–59, 133–135.
Scatchard, G., and Bregman, J. (1959), *J. Am. Chem. Soc. 81*, 6095.
Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., and Van Holde, K. E. (1962), *J. Biol. Chem. 237*, 1107.
Sophianopoulos, A. J., and Van Holde, K. E. (1964), *J. Biol. Chem. 239*, 2516.
Steiner, R. F. (1953), *Arch. Biochem. Biophys. 44*, 121; *47*, 59.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford, Clarendon, pp 57, 305.
Townend, R., and Timasheff, S. N. (1960), *J. Am. Chem. Soc. 82*, 3168.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Wales, M. (1948), *J. Phys. Colloid Chem. 52*, 235.
Yphantis, D. (1964), *Biochemistry 3*, 297.

Ultracentrifuge Investigation of Protein Aggregation in Dilute Solutions of C-Phycocyanin*

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ABSTRACT: Sedimentation analysis of dilute solutions of phycocyanin with absorption optics demonstrated that aggregates previously detected at concentrations up to 40 mg/ml are present at concentrations as low as 0.2 mg/ml at pH 5–9. The S values of the various aggregates also agree with results in previously reported work with concentrated solutions and therefore permit the interpretation of absorption measurements and electron microscopy in terms of these aggregates. Underexposure of photographic plates with the absorption optics on the ultracentrifuge is characterized as being responsible for the probable inability of Hattori *et al.* [Hattori, A., Crespi, H. L., and Katz, J. J. (1965), *Biochemistry 4*, 1225] to detect higher aggregates. The photographic absorption technic, therefore, requires close scrutiny of exposures for proper application.

In previous reports, Scott and Berns (1965; Berns and Scott, 1966) demonstrated that C-phycocyanin from several *Cyanophyta* aggregates reversibly. The aggregation was studied in the concentration range of 4–50 mg/ml as a function of temperature, pH, and ionic strength. Reversible aggregation of this protein was characterized as being sensitive to the use of cellulose ion exchangers and certain types of calcium phosphate preparations (Scott and Berns, 1965). Aggregates sedimenting at 11 S and higher were described as being important *in vivo*. The 11S material was suggested to be a hexamer and hydrodynamic measurements and electron microscopy (Berns and Edwards, 1965) agreed quite well in delineating the probable size and shape of this aggregate. Most of the physical measurements in these studies have been at concentrations of 5 mg/ml and greater; however, spectrophotometric and electron microscopy studies were generally confined to concentrations in the 0.5-mg/ml region and lower. The question arises, therefore, as to whether absorption spectra can be interpreted in terms of the aggregates proposed from work with higher concentrations and are the electron microscopy results relevant to the species observed at much higher concentration. If simple equilibria exist, then a natural consequence, judging from the published data for this system, should be an increase in concentration of the slower sedimenting species as the total protein concentration is decreased. Hattori *et al.* (1965) have reported that in ultracentrifugation of dilute solutions the slower sedimenting 3S material is favored and in most cases the 7S or 11S species is totally absent. These studies were performed with phycocyanin purified by ion-exchange chromatography and, therefore, the aggregation phenomenon as investigated is an artifact (Scott and Berns, 1965). Even in this type of system, however, immunochemical techniques (Scott and Berns, 1965) detect the presence of higher aggregates. Ouchterlony double diffusion utilizes antigen concentrations of 0.5 mg/ml and lower. A serious inconsistency that bears quite strongly on studies of

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