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SEDIMENTATION COEFFICIENTS OF SELF-ASSOCIATING SPECIES

II. TESTS WITH A SIMULATED EXAMPLE AND WITH β-LACTOGLOBULIN A

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If sedimentation equilibrium and sedimentation velocity experiments are performed on a self-associating solute under the same solution conditions, it is possible to evaluate the sedimentation coefficients (s_i) of the self-associating species and the usual concentration dependence parameter (g or g_s). We have tested some of these methods with simulated examples. A more critical test is to use real data. Sedimentation equilibrium experiments with β-lactoglobulin A at 20°C in 0.2 M glycine buffer (pH 2.46) indicated that a nonideal monomer-dimer association was present. Sedimentation velocity experiments were performed on β-lactoglobulin A under the same conditions. Using data from both sets of experiments we were able to evaluate s_1, s_2, g and g_s using two different models for s_{wa}, the apparent weight average sedimentation coefficient. The empirical model for s_{wa} developed by Weirich et al. [1] gave better variance than did the model for s_{wa} developed by Gilbert and his co-workers [2–5]. Using a simulated monomer-dimer association mimicking a system having higher sedimentation coefficients than β-lactoglobulin A did, we were able to show that one could not obtain s_2 from tangents to the plot of $1/s_{wa}$ vs. c in the high concentration region. The methods developed here for sedimentation coefficients can be applied to other experiments in which a weight average property (or its apparent value) of a self-associating solute is measured, provided the appropriate thermodynamic experiments are done under the same solution conditions.

1. Introduction

In an earlier publication [1] we have shown how one can evaluate sedimentation coefficients of self-associating solutes, provided that sedimentation velocity experiments and appropriate thermodynamic experiments (elastic light scattering, sedimentation equilibrium, osmotic pressure) are performed under identical solution conditions. If it is assumed that interacting (coupled) flows are absent, then one can obtain the sedimentation coefficient of the self-associating species and also the usual sedimentation concentration dependence parameter from a combination of these experiments. Furthermore, one can also estimate the translational diffusion coefficients of the self-associating species from these experiments.

Here we will show some results obtained with a simulated example, which simulates the situation one might encounter with relatively fast sedimenting solutes. We will also show some results obtained with real experiments on a slower sedimenting molecule, β-lactoglobulin A.

2. Tests with a simulated example

Before beginning the experimental tests, we used a simulated monomer-dimer association as an example. We wanted to see how well and how sensi-

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tive the previously developed methods for obtaining the sedimentation coefficients and the concentration dependence parameter (g or g_s) would be. Two empirical models have been developed to describe s_{wa} for a self-associating system. Model I, proposed by Weirich et al. [1], is described by

$$1/s_{wa} = 1/s_{wc} + g_s c \quad \text{(model I)}. \tag{1}$$

Here

$$s_{wc} = \Sigma c_i s_i / c \quad (i = 1, 2, \ldots) \tag{2}$$

is the weight average sedimentation coefficient and g_s the concentration dependence parameter of s_{wa}. Model II, proposed by Gilbert and his associates [2–5], is defined by

$$s_{wa} = s_{wc}/(1 + gc) \tag{3}$$

or

$$1/s_{wa} = 1/s_{wc} + (g/s_{wc})c. \tag{4}$$

Here s_{wc} is defined by eq. 2 and g is the concentration dependence parameter of s_{wa}. If sufficient data are available in the low solute concentration region, then one can obtain s_1 from extrapolation of a plot of $1/s_{wa}$ vs. c or of s_{wa} vs. c, since

$$\lim_{c \to 0} (1/s_{wa}) = 1/s_1 \tag{5}$$

or

$$\lim_{c \to 0} s_{wa} = s_1. \tag{6}$$

For the simulated example we used an example based on model I and having the following characteristics: $s_1 = 7.2$ S (1 S = 1 Svedberg unit = 10^{-13} s), $s_2 = 10.18$ S, $g_s = 0.005$ l g^{-1} s^{-1} and $k_2 = 0.65$ l/g. With model I there are three methods for obtaining s_2 and g_s; these methods are described in a preceding publication [1]. Fig. 1 shows plots of $1/s_{wa}$ vs. c for three choices of g_s, including the one used in table 1. Note that as g_s increases from 0 to 0.01, the shape of the curve changes considerably. With positive values of g there is always a minimum in the plots of $1/s_{wa}$ vs. c. This behavior is similar to that encountered with plots of M_1/M_{wa} vs. c (or of M_1/M_{na} vs. c) for nonideal monomer-dimer self-associations. The similarity of these plots was really the basis for advocating model I.

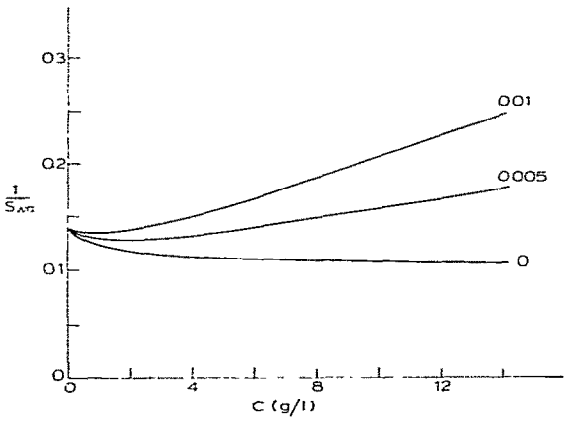

Fig. 1. Synthetic data curves generated from model I at several g_s values. For this example $s_1 = 7.20$, $s_2 = 10.18$ and $k_2 = 0.65$ l/g. g_s values are given by each curve.

Table 1 shows the ability of each of the three methods of model I to analyze for the proper value of s_2 for the hypothetical monomer-dimer association. Synthetic $1/s_{wa}$ values were generated from

$$1/s_{wa} = 1/s_{wc} + g_s c = \frac{1 + k_2 c_1}{s_1 + k_2 c_1 s_2} + g_s c \tag{7}$$

for a monomer-dimer association. The appropriate value of c_1, the monomer concentration in g/l, at each concentration was determined from

$$c_1 = \left(-1 + (1 + 4k_2 c)^{1/2}\right)/2k_2 \tag{8}$$

since $c = c_1 + k_2 c_1^2$. The $1/s_{wa}$ quantity was manipulated according to the various methods to give the value given as 'x_{true}.' The quantity 'x_{calc}' is a function only of c_1, k_2, s_1 and s_2. If k_2, c_1 and s_1 are known then s_2 may be found using successive approximation techniques until x_{calc} equals x_{true}. Convergence to the 'true' value of s_2 (10.18 S) at several values for c equal to 4 and 12 g/l is also shown in table 1. It is seen that there is good agreement for the value of s_2 obtained by all three methods.

Table I

Evaluation of s_2 by various methods

$s_2 = 10.18$ S [a], $s_1 = 7.2$ S [a], $g_s = 0.005$ l g^{-1} s^{-1}, $k_2 = 0.65$ l/g, model I.

Method	Relation		Choice of s_2 for x_{calc}	Convergence at 4 g/l		Convergence at 12 g/l	
	x_{true}	x_{calc}		x_{true}	x_{calc}	x_{true}	x_{calc}
1 [b]	$\frac{1}{s_{wa}} c \frac{d}{dc} \frac{1}{s_{wa}}$	$= \frac{1}{s_{wc}} c \frac{d}{dc} \frac{1}{s_{wc}}$	8	0.1195	0.1332	0.1125	0.1305
			9	0.1195	0.1266	0.1125	0.1213
			10.18	0.1195	0.1196	0.1125	0.1119
			11	0.1195	0.1151	0.1125	0.1062
2 [c]	$\frac{1}{s_{wa}}$	$= \frac{1}{s_{wc}} - c \frac{d}{dc} \frac{1}{s_{wc(c-c^*)}}$	8	0.1334	0.1375	0.1677	0.1483
			9	0.1334	0.1355	0.1677	0.1578
			10.18	0.1334	0.1330	0.1677	0.1664
			11	0.1334	0.1311	0.1677	0.1710
3	$\frac{1}{s_{wa}} - \frac{2}{c} \int_0^c \frac{dc}{s_{wa}}$	$= \frac{1}{s_{wc}} - \frac{2}{c} \int_0^c \frac{dc}{s_{wc}}$	8	−0.1268	−0.1354	−0.1188	−0.1329
			9	−0.1268	−0.1312	−0.1188	−0.1260
			10.18	−0.1268	−0.1266	−0.1188	−0.1188
			11	−0.1268	−0.1235	−0.1188	−0.1143

[a] Svedberg unit (S) = 10^{-13} s.
[b] Methods I and IV of ref. 1 are identical.
[c] $c = c^*$ at the minimum of the $1/s_{wa}$ vs. c plot.

3. Sedimentation velocity of β-lactoglobulin A at pH 2.46

In 0.2 M glycine buffer (0.2 M glycine, 0.1 M HCl, $I = 0.1$, pH 2.46) at 20°C, β-lactoglobulin A undergoes a reversible, monomer-dimer self-association. This temperature-dependent self-association has been studied by Tang and Adams [6] using sedimentation equilibrium experiments. At 20°C the values of the association constant (k_2) and the nonideal term (BM_1) were $k_2 = 0.350$ l/g and $BM_1 = 0.0131$ l/g. The protein solutions used in the sedimentation velocity experiments were prepared as described previously. Concentrations were determined by differential refractometry at a wavelength of 546 nm for concentrations from 3.88 to 11.9 g/l; the refractive index increment was assumed to be 1.82×10^{-4} l/g [6,7]. For concentrations below 3.88 g/l the absorbance at 280 nm was used. Sedimentation velocity experiments were performed on Beckman Model E Analytical Ultracentrifuges at 60000 rpm. Both ultracentrifuges were equipped with electronic speed controls and a resistance-temperature indicator control, so that temperature could be controlled to better than ±0.1°C. The experiments on solutions having lower concentrations (3.26 g/l or lower) were performed on an ultracentrifuge equipped with a photoelectric scanner and multiplexer; the wavelength used was 280 nm. For greater concentrations an ultracentrifuge equipped with Rayleigh and schlieren optics was used. The schlieren patterns were obtained at various times at a wavelength of 546 nm.

For the experiments performed with a photoelectric scanner the initial concentrations were determined as follows: First a stock solution was prepared and dialyzed against the glycine buffer;

its concentration was determined by differential refractometry at $\lambda = 546$ nm. Careful dilutions were prepared from the stock solution, so that the concentrations of these working solutions could be related to the stock solution. The initial concentrations of these working solutions were also determined by measuring the pen deflections on the chart recorder of the photoelectric scanner; these measurements were done at 280 nm. This way the pen deflections could be related to the concentrations in fringes at 546 nm.

Fig. 2 shows a plot of the apparent weight average sedimentation coefficients (s_{wa}) vs. the average total protein concentration (c_{av}). Because β-lactoglobulin A is a slowly sedimenting molecule, the moving boundary does not break away readily from the radial position of the air/solution meniscus, r_m, and it was necessary to use the second moment method, as modified by Baldwin [8] and by Webber [9], for the evaluation of s_{wa} from the schlieren data. Here one notes that

$$s_{wa} = \frac{1}{2\omega^2}\frac{\mathrm{d}\ln\langle r^2\rangle}{\mathrm{d}t} \tag{9}$$

and $\langle r^2\rangle$ is obtained from [10]

$$\langle r^2\rangle = (1/c_p)\left[c_m r_m^2 + \int_{r_m}^{r_p} r^2(\partial c/\partial r)_t \mathrm{d}r\right]. \tag{10}$$

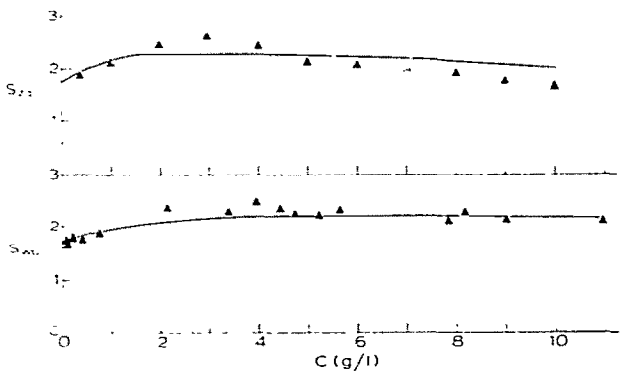

Fig. 2. Regenerated fit (solid lines) to β-lactoglobulin A data by model I, method 3.

These quantities have their usual meanings: ω is the angular velocity of the rotor ($\omega = 2\pi(\mathrm{rpm})/60$); $\langle r^2\rangle$ is the square of the boundary position which moves with the same velocity as do particles in the plateau region – region where $(\partial c/\partial r)_t = 0$; c_p is the concentration of β-lactoglobulin A in the plateau region; c_m is the concentration of β-lactoglobulin A at r_m, the radial position from the center of rotation of the air/solution meniscus; r is any radial position between r_m and r_p, the radial position at which the plateau begins; and t is the time. The meniscus concentration, c_m, is evaluated from the well known equation [10]

$$c_m = c_0 - (1/r_m^2)\int_{r_m}^{r_p} r^2(\partial c/\partial r)_t \mathrm{d}r. \tag{11}$$

Here c_0 is the original concentration of β-lactoglobulin A. The plateau concentration is obtained from

$$c_p = c_m + \int_{r_m}^{r_p}(\partial c/\partial r)_t \mathrm{d}r. \tag{12}$$

The value of s_{wa} obtained from a plot based on eq. 9 reflects the value of s_{wa} for the average concentration (c_{av}) of β-lactoglobulin A during the sedimentation velocity experiment. This value of c_{av} is estimated from [11]

$$c_{av} = (c_0/r_m^2)\frac{\langle r^2\rangle_1 + \langle r^2\rangle_n}{2} \tag{13}$$

where $\langle r^2\rangle_1$ and $\langle r^2\rangle_n$ refer to the values of $\langle r^2\rangle$ for the first and last photographs, respectively.

In the lower concentration range, the values of s_{wa} in fig. 2 were determined from experiments using a photoelectric scanner at a wavelength of 280 nm. Here s_{wa} was determined from the change in the logarithm of the plateau concentration with time according to [1,11]

$$\frac{\mathrm{d}\ln c_p}{\mathrm{d}t} = -2\omega^2 s_{wa}. \tag{14}$$

The plot of s_{wa} vs. c was analyzed by the three methods of model I. The results of this analysis are shown in table 2 and refer to the values in 0.2 M glycine buffer at pH 2.46.

The value of s_1 (1.68 S) was obtained by extrapolation of the s_{wa} vs. c data to $c = 0$. For methods employing derivatives a value of s_2 was

Table 2

Model	Method	s_2 (S)	g_s (l g^{-1} s^{-1}) or g (l g^{-1})	Variance ($\times 10^4$)
I	1	2.78 ± 0.09	0.0021 ± 0.005	5.46
I	2	3.11 ± 0.06	0.0064 ± 0.003	2.54
I	3 [a]	3.15 ± 0.08	0.0068 ± 0.003	2.38
II	1	3.17 ± 0.03	0.0179 ± 0.008	2.57
II	2	2.47 ± 0.03	−0.005 ± 0.01	10.6
II	3	2.37 ± 0.07	−0.009 ± 0.02	12.9

[a] Gave the best fit.

determined at increments of 0.02 g/l from the smoothed experimental curve over the entire concentration range. For models I and II, method 3, which required that an integral be evaluated, s_2 was determined only for integrals which covered at least one-third of the concentration range. Acceptable solutions for s_2 by all methods were restricted to be between s_1 and $2s_1$. A best value of s_2 for each method was obtained through linear regression analysis of the s_2 values found at each concentration. This value was then used to regenerate the ideal weight average sedimentation coefficient (see eq. 7). The concentration dependence parameter was calculated from

$$g_s = \left(\frac{1}{s_{wa(exp)}} - \frac{1}{s_{wc(calc)}} \right) / c \qquad \text{for model I} \tag{15}$$

or

$$g = \left(\frac{s_{wc}}{s_{wa}} - 1 \right) / c \qquad \text{for model II.} \tag{16}$$

This parameter value from regression analysis was combined with the calculated $1/s_{wc}$ quantities to give a regenerated $1/s_{wa}$ vs. c curve. The variance between the experimental and regenerated curves was used as the criterion for determining which model gave the best fit. Method 3 might be expected to give a slightly better fit than the other methods, since numerical integration is usually a more precise procedure than is differentiation. This is indeed the case as is seen in table 2. Regenerated values of s_{wa} vs. c from method 3 of model I are shown by the solid line in fig. 2. In addition a plot of s_{za} vs. c is also shown in fig. 2; here [1,12].

$$s_{za} = \mathrm{d}(cs_{wa})/\mathrm{d}c. \tag{17}$$

The smooth curve was drawn from values of s_{za} regenerated from the results of method 3 of model I and the relation

$$s_{za} = \frac{s_1 + 2k_2c_1s_2}{(1+2k_2c_1)\left(1 + g_sc\dfrac{s_1 + k_2c_1s_2}{1+k_2c_1}\right)^2}. \tag{18}$$

The experimentally derived quantities for s_{za} were calculted by a five-point derivative procedure from the smoothed s_{wa} data.

The value of s_1 corresponds to s_1°, the value of s_{wa} at infinite dilution, and the value of s_2, since the usual concentration dependence of s_{wa} vs. c has been corrected, would correspond to s_2°. Thus, these values of s_1 and s_2 can be inserted into the appropriate form of the Svedberg equation [13],

$$s_i = \frac{iM_1(1-\bar{v}\rho)}{Nf_i} = \frac{iM_1(1-\bar{v}\rho)D_i}{RT} \qquad (i = 1, 2) \tag{19}$$

to give the translational diffusion coefficient D_i of the self-associating species i. Here, as in the sedimentation equilibrium experiments, it has been assumed that all partial specific volumes are equal ($\bar{v}_i = \bar{v}_2 = \bar{v}$). One can also obtain the translational frictional coefficient, f_i, since [13]

$$D_i = \frac{RT}{Nf_i}. \tag{20}$$

The values for f_i and D_i are listed in table 3.

Table 3

Translational frictional and diffusion coefficients at 20°C in glycine buffer

$f_{i,min} = 6\pi\eta r_i$ (Stokes' law), $f_i = M_i(1-\bar{v}\rho)/Ns_i$, $r_i = (0.75M_i\bar{v}/N\pi)^{1/3}$, $N = 6.023\times10^{23}$ (Avogrado's number), $D_i = RT/Nf_i$.

Species	s_i (s) ($\times10^{13}$)	D_i (cm^2/s) ($\times10^7$)	f_i (g/s) ($\times10^8$)	$f_i/f_{i,min}$
Monomer	(1.68 ± 0.05)	(9.0 ± 0.3)	(4.5 ± 0.1)	1.34 ± 0.04
Dimer	(3.15 ± 0.08)	(8.4 ± 0.2)	(4.8 ± 0.1)	1.14 ± 0.03

4. Discussion

We have shown with a simulated example based on model I that the methods we developed for obtaining sedimentation coefficients and the usual concentration dependence parameter for sedimentation work with the three models tested. Method 3, since it is based on integration which is a smoothing process, may be the method of choice in real experiments. Since model II is different from model I, one would expect to get different values of s_2 and g (or g_s) with each model. Both models are empirical models, and one cannot necessarily say which is the more favored one. Both models use only one sedimentation coefficient concentration dependence parameter (g or g_s). Soda et al. [14] did a very elegant study of the Johnston-Ogston effect with poly(α-methylstyrene) samples in a good solvent (toluene) and in a poor solvent (cyclohexane) at 35°C. Using a blend of two sharp fractions they found that the sedimentation coefficient concentration dependence parameter of the weight average sedimentation coefficient was simply the weight average of the two individual concentration dependence parameters, i.e.,

$$g = (g_{11}c_1^0 + g_{22}c_2^0)/c^0 \tag{21}$$

where

$$c^0 = c_1^0 + c_2^0 \tag{22}$$

is the total concentration of polymer. There were no cross-terms, i.e., g_{12} or g_{21} were essentially negligible within the precision of their experiments. For a self-associating solute it would be virtually impossible to measure g_{11} and g_{22}, so if one assumes $g_{11} = g_{22}$, then only one sedimentation coefficient concentration dependence parameter is needed. Soda et al. used an analog of model II.

For some monomer-n-mer associations, those with $n \geq 3$, there may be some separation of the boundaries due to monomer and to n-mer, if the molecular weight of the n-mer is high enough; otherwise, the effects of diffusion will mask the separation [15,16]. When separation of the moving boundaries does occur, one may be able to evaluate s_1 and s_n from the boundaries. For monomer-dimer-trimer (1,2,3), monomer-dimer-tetramer (1,2,4) and various indefinite self-associations, separation of the boundaries does not usually occur [15,17,18], so that one would have to resort to procedures described in the earlier paper [1] to evaluate the various sedimentation coefficients and the concentration dependence parameter.

The plot of s_{wa} vs. c for β-lactoglobulin A in 0.2 M glycine buffer at 20°C shown in fig. 2 is characteristic of some self-associations [19,20]. In our experiments we analyzed the data using both models, since these are actual experimental data. Note that there is some scatter in the s_{wa} vs. c data with this slowly sedimenting system, and this may be a characteristic of systems having relatively low sedimentation coefficients, since this effect has been observed by others with β-lactoglobulin A at low pH [22], and with chymotrypsinogen [19]. Kakiuchi and Williams [20] studied the monomer-dimer association of a papain-digested fragment of immunoglobulin Ig in 8 M urea; they encountered much larger sedimentation coefficients and seemed to have better precision. On the other hand, they had to obtain their estimate of s_1 by performing a separate experiment under conditions where no self-association occurred. Townend et al. [22] re-

ported that β-lactoglobulin (sic) had an s value of approx. 1.9 S at zero concentration at pH 1.6. They used schlieren optics. It is not known from their paper whether they used the general form of the second moment method or if they used boundary-forming experiments for calculating s_{wa} values. Because the monomer is such a slowly sedimenting molecule, the moving boundary may not go to the baseline near the air/solution meniscus (it did not in our experiments). Thus, the Faxén solution to the continuity equation would not be approximated, and the s values can be erroneous – they can be larger than they should be [23,24].

The results given by the two models for s_{wa} (see eqs. 1 and 3) are shown in table 2. It is evident that, in general, consistently better variances are obtained with model I. The values of s_2 from both models are close to each other. From the values of s_1 and s_2 one can calculate the translational diffusion coefficients D_1 and D_2 (see eq. 19) and also the frictional coefficients f_1 and f_2 (see eq. 20). These values are listed in table 3. For the monomer one can calculate f_1/f_{1min}, where $f_{1min} = 6\pi\eta r_1$ is the Stoke's law frictional coefficient for a sphere. Since $f_1/f_{1min} = 1.34$, the monomer is not spherical. Using the best value for s_2 one can show that $f_2/f_{2min} = 1.14$ and that $s_2/s_1 = 1.88$; this last ratio would be $2^{2/3}$ or 1.59, if both particles were spheres. The frictional ratio (f_1/f_{1min}) for the monomer clearly indicates that it is not a sphere, and the the frictional ratio for the dimer $f_2/f_{2min} = 1.14$ indicates less deviation from a sphere. This may suggest that the dimer forms a side-to-side compact molecule rather than an elongated (perhaps end-to-end) one.

We have calculated the value of s_{zapp}. A plot of s_{zapp} vs. c for β-lactoglobulin A is shown in fig. 2; this may well be the first attempt to evaluate s_{zapp} experimentally. Differentation is a noisy process and can produce scatter, so the regenerated fit to s_{zapp} is fair. One might have had better results if the s_{wapp} value could have been more precise. It can be shown for model I that methods 1 and 4 of the previous paper [1] are identical; furthermore, method 1 involves s_{za}. This may account for the higher variance and lower value for s_2 (see table 2) with this method. It should be noted that methods 2 and 3 give good agreement with each other. Method 3 is based on integration which is a smoothing process, and method 2 takes advantage of the fact that $d(1/s_{wa})/dc = 0$ at the minimum in the plot of $1/s_{wa}$ vs. c.

At high concentrations eq. 7 becomes

$$\frac{1}{s_{wapp}} = \frac{1}{s_2}\frac{\left[1+\frac{1}{k_2c_1}\right]}{(1+[s_1/s_2]/k_2c_1)} + g_s c \cong \frac{1}{s_2} + g_s c. \qquad (23)$$

Thus, attempts have been made to extrapolate a tangent line to the $1/s_{wapp}$ vs. c plot taken at high concentrations, and estimates of s_2 were made from the intercept at zero concentration. We tried this procedure with the perfect data shown in fig. 1. Tangents were taken at various parts of the high concentration region, and in no case did we recover the value of s_2. The value of s_2 ranged from 8.84 to 8.98 S. The best value we got was 88% of the true s_2 value. Thus, this method at best can only give a ball-park estimate of s_2. The tediousness associated with the evaluation of schlieren or Rayleigh data needed for the second moment calculation might be overcome by using an automated plate reader [25–27].

A referee has pointed out that if strong pressure effects were present, then there would no longer be a plateau region of constant composition, and in the case of dissociation, the total concentration would rise above the original [28]. In this case neither the Gilbert theory nor the second moment method would apply. Indeed, it would be difficult to apply this analysis to an associating protein, such as myosin [28–30], which shows a pronounced pressure effect. In our experiments, the plateau and the solvent (buffer solution) baseline coincided, which would indicate no pressure effects. Our experiments were terminated before the plateau disappeared.

We have shown that one can use sedimentation equilibrium and sedimentation velocity experiments performed under identical solution conditions to evaluate the sedimentation coefficient of the dimer (s_2) as well as the usual concentration dependence of the sedimentation coefficient (g or g_s). The procedures used here can be applied to other experiments that give a weight (x_{wc}) or apparent weight average property (x_{wapp}), such as

elution volume, to evaluate the individual properties x_i and the usual concentration dependence of x, provided that the appropriate thermodynamic experiments have been performed so that c_1 and k_i are known. It should also be noted that x_{zc} or x_{zapp} is also available [1], since

$$x_{zapp} = \mathrm{d}(cx_{wapp})/\mathrm{d}c. \quad (24)$$

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