55, 181.
Murdock, A. L., and Koeppe, O. J. (1964), *J. Biol. Chem. 239*, 1983.
Nemethy, G., and Scheraga, H. A. (1964), *J. Chem. Phys. 41*, 180.
Noel, J. K. F., and Schumaker, V. N. (1972), *J. Mol. Biol. 68*, 523.
Pickels, E. G., Harrington, W. F., and Schachman, H. K. (1952), *Proc. Nat. Acad. Sci. U. S. 38*, 943.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Richards, E. G., Teller, D., and Schachman, H. K. (1971), *Anal. Biochem. 41*, 189.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.
Schachman, H. K. (1963), *Biochemistry 2*, 887.
Schumaker, V. (1968), *Biochemistry 7*, 3427.
Schumaker, V., and Adams, P. (1968), *Biochemistry 7*, 3422.
Simon, I. (1972), *Eur. J. Biochem. 30*, 184.
Smith, G. D., Kirschner, M. W., and Schachman, H. K. (1973), *Biochemistry 12*, 3801.
Smith, G. D., and Schachman, H. K. (1972), *Proc. Austr. Biochem. Soc. 5*, 13.
Steinberg, I. Z., and Schachman, H. K. (1966), *Biochemistry 5*, 3728.
Taylor, J. F., and Lowry, C. (1956), *Biochim. Biophys. Acta 20*, 109.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 164*, 66.
Vas, M., and Boross, L. (1970), *Acta Biochim. Biophys. Acad. Sci. Hung. 5*, 215.
Velick, S. F. (1955), *Methods Enzymol. 1*, 401.
Velick, S. F., Baggott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10*, 779.
Velick, S. F., and Furfine, C. (1963), *Enzymes 1, 2nd Ed.*, 243.
Velick, S. F., Hayes, J. E., Jr., and Harting, J. (1953), *J. Biol. Chem. 203*, 527.
Watson, H. C., Duée, E., and Mercer, W. D. (1972), *Nature (London), New Biol. 240*, 130.
Winer, A. D. (1964), *J. Biol. Chem. 239*, PC 3598.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zavodszky, P., Abaturov, L. B., and Varshavsky, Y. M. (1966), *Acta Biochim. Biophys. Acad. Sci. Hung. 1*, 389.

Analysis of Association–Dissociation Equilibria in Proteins by Difference and Differential Sedimentation†

Geoffrey D. Smith, Marc W. Kirschner, and H. K. Schachman*

ABSTRACT: Although the weight average sedimentation coefficient, s, of an oligomeric protein is affected frequently by the addition of specific ligands, it is not clear generally whether this effect is due to a shift in the association–dissociation equilibrium of the protein or to a conformational change leading to an altered frictional coefficient. This ambiguity can be eliminated, in part, by combining measurements of the change in sedimentation coefficient, Δs, at one protein concentration with data for the effect of the ligand on the concentration dependence of the sedimentation coefficient, ds/dc. Equations are presented relating Δs at a single protein concentration to a ligand-promoted shift in the association constant for a monomer–dimer system. Analogous equations are derived relating the association constant (and changes in it) to the observed values of ds/dc. Two types of self-associating systems are considered: in one the extent of association is very high with the protein being mainly in the dimeric form; for the other the protein is largely in the dissociated form as monomer. The theoretical treatments show how these two types of system differ with regard to the values of Δs and the changes in ds/dc when ligands perturb a monomer–dimer equilibrium. Extremely small changes in Δs can be measured accurately and directly by the recently developed difference sedimentation technique based on interference optics. Accurate measurements of ds/dc are readily obtained by the differential sedimentation technique in which differing concentrations of the same protein solution are layered over one another in a synthetic boundary cell. The assumptions and limitations inherent in the use of these sedimentation velocity techniques for detecting and measuring shifts in the self-association of proteins are discussed in relation to the theoretical treatment.

Many proteins are known to undergo conformational changes in response to the binding of substrates and ligands, and much work is being devoted to the determination of the nature and magnitude of these changes. Such effects are manifested either as alterations in the secondary and tertiary structures of the individual chains themselves or in the state of aggregation of these chains within oligomers, or to a combination of both factors. Among the many physical-chemical techniques used for examining such changes, hydrodynamic methods such as sedimentation velocity have been found to be particularly fruitful since the measured parameter, the sedimentation coefficient, is sensitive to both the molecular weight and the shape of the kinetic unit (Svedberg and Pedersen, 1940).

† From the Department of Molecular Biology and Virus Laboratory, Wendell M. Stanley Hall, University of California, Berkeley, California 94720. *Received July 25, 1972.* This research was supported in part by U. S. Public Health Service Research Grants GM 12159 from the National Institute of General Medical Sciences and AI 01267 from the National Institute of Allergy and Infectious Diseases and by National Science Foundation Research Grant GB 4810X.

When the conformational changes in oligomeric proteins are large, as in the denaturation by urea or guanidine hydrochloride for example, there is little difficulty in distinguishing a decrease in molecular weight from changes in shape or volume by the sedimentation velocity technique. However, with the advent of ultracentrifugal methods (Richards and Schachman, 1957; Kirschner and Schachman, 1971a) sensitive enough to detect very small changes in sedimentation coefficient of the magnitude produced by the binding of ligands to enzymes, the problem of distinguishing changes in shape or volume from changes in the state of association of the subunits becomes much more difficult. Although both types of effects justifiably may be termed "conformational changes," the distinction between the two is of obvious significance. Goers and Schumaker (1970) considered this problem in studying hemoglobin, where oxygenation apparently causes changes both in the association and in the conformation of individual polypeptide chains. In this case they were able to employ association constants previously determined for the different oxygenation states of hemoglobin to estimate the contribution of a shift in the association–dissociation equilibria to their measured differences in sedimentation coefficient. A different approach was employed by Kirschner and Schachman (1971b) in an attempt to determine whether protein association contributed to the increase in sedimentation coefficient observed upon the binding of ligands to the catalytic subunit of aspartate transcarbamylase. They performed simultaneous differential sedimentation experiments with a synthetic boundary cell to measure the concentration dependence of the sedimentation coefficient of the catalytic subunit in the presence and absence of effective ligands; analysis of the results indicated that there was no change in the state of aggregation on binding ligands and that the measured differences in sedimentation coefficient at a fixed protein concentration could be ascribed to changes in the shape or volume of the subunit.

In the present communication we discuss the application of these two currently available techniques, *viz.*, difference sedimentation (Richards and Schachman, 1957; Kirschner and Schachman, 1971a,b; Schumaker and Adams, 1968) and differential sedimentation (Hersh and Schachman, 1955), to the problem of evaluating the contribution of changes in state of association to measured differences in sedimentation coefficient. Although it is customary to use sedimentation equilibrium to measure molecular weights and changes thereof, the changes in molecular weight due to shifts in association–dissociation equilibrium are frequently so small for certain systems that they cannot be detected or at least measured accurately by the sedimentation equilibrium technique. For such systems the use of the sedimentation velocity techniques of difference and differential sedimentation may prove fruitful.

The treatment presented here is an extension of that introduced by Gilbert (1960) who first pointed out the effect of association–dissociation equilibria on the concentration dependence of the sedimentation coefficient. These considerations were applied by Gilbert and Gilbert (1961, 1962) in analyzing data for the dissociation and aggregation of β-lactoglobulin.

In the treatment presented here use is made of the demonstration (Gilbert, 1960) that a system undergoing association or dissociation shows a dependence of the sedimentation coefficient on concentration over and above the usual hydrodynamic effects for a single substance. This effect, which can be measured accurately by the differential sedimentation technique, is combined with measurements of small changes in sedimentation coefficients by difference sedimentation. Equations are derived to show that by measuring the difference in sedimentation coefficient at various concentrations of the two solutions, or by measuring the difference in the concentration dependence of the sedimentation coefficients of each solution, it is possible to estimate quantitatively the contribution of changes in degree of association to measured conformational changes. In this way changes in shape (or volume) without concomitant alterations in molecular weight can be detected and estimated.

General Considerations

The sedimentation coefficient, s, of macromolecules is related both to their molecular weight and to their frictional coefficient (Svedberg and Pedersen, 1940). A change in the sedimentation coefficient, accompanying the addition of a ligand, is therefore attributable, on the one hand, to a change in the effective molecular weight, due to an alteration in the state of association or the slight change in the weight and partial specific volume accompanying binding, or, on the other hand, to a change in the frictional coefficient, stemming from alterations in the shape or volume of the hydrodynamic unit. These considerations apply to infinitely dilute solutions; additional complications arise from the effect of concentration on the sedimentation coefficient of the macromolecules. For globular proteins which do not exhibit association–dissociation equilibria the sedimentation coefficient decreases slightly, and almost linearly, with concentration due to viscosity, density, and backward flow effects (Schachman, 1959).

Now let us consider the effect of concentration of the sedimentation coefficient of associating–dissociating systems. When macromolecules exist in equilibrium with their subunits or aggregates, the sedimentation of the mixture may be characterized by a weight-average sedimentation coefficient, $\bar{s}$, which is the average of the sedimentation coefficients of all components in terms of their weight concentrations in solution. For a monomer–dimer system Gilbert (1955) has shown that a single sedimenting boundary is observed at all concentrations if the interconversion from monomer to dimer (and *vice versa*) is very rapid compared to the rate of separation of monomer and dimer. Such systems are revealed in sedimentation velocity experiments at different concentrations by a characteristic concentration dependence of $\bar{s}$ as first illustrated with α-chymotrypsin (Schwert, 1949). At a low protein concentration, where monomers predominate, the measured $\bar{s}$ is characteristic of the monomer s value. Increasing the concentration leads to an increase in $\bar{s}$ as the chemical equilibrium is shifted toward the dimer. When the concentration is sufficiently high, as dictated largely by the equilibrium constant, the $\bar{s}$ approaches a maximum after which further increasing the concentration leads to a gradual decrease in $\bar{s}$ due to the effect of concentration on the aforementioned hydrodynamic factors. Extrapolation in a linear fashion of data obtained at high concentrations to infinite dilution has been used to give an approximate value of the sedimentation coefficient of the dimer.

If the association constant is large, the maximum of the $\bar{s}$ *vs.* c curve is reached at low protein concentrations; hence over the concentration range of interest, or over the readily accessible range of concentration, the sedimentation characteristics of the mixture may resemble those of dimer alone. This type of system (case I) is illustrated in Figure 1, where the line with alternate long and short dashes represents the concentration dependence of the s of pure dimer and that

with short dashes represents pure monomer with a normal concentration dependence of s in each case. The solid line shows the calculated curve of s *vs.* c for an equilibrium mixture having a monomer–dimer association constant of 100 l./g. As seen for case I in Figure 1, a linear extrapolation of the data for the monomer–dimer system from high concentrations to infinite dilution does not yield the correct value for the sedimentation coefficient of the dimer; on the contrary, a somewhat lower value is obtained (Gilbert, 1960; Gilbert and Gilbert, 1961).

Alternatively for a monomer–dimer system of very low equilibrium constant, the system may be predominantly in the dissociated form throughout the experimentally accessible range of concentrations. For such cases it is possible for the hydrodynamic contribution to the s *vs.* c dependence to be predominant and the initial increase in s with increasing concentration is not manifested. Such a situation is illustrated by case II in Figure 1. In this case the line with alternate long and short dashes represents the dependence on concentration of the s of pure monomer and the line with long dashes represents that of dimer alone. The solid line (case II) represents the monomer–dimer equilibrium mixture and was calculated for an association constant of 0.01 l./g.

For both cases I and II, as illustrated in Figure 1, three important features are worthy of comment. First, at any given concentration a shift in the association–dissociation equilibrium results in a change in the sedimentation coefficient. Second, over a large concentration range in each case the dependence of s on c departs only slightly from linearity and is negative. Third, although there is a negative s *vs.* c dependence in both cases the slope of the curve describing this negative dependence is reduced relative to that of the line describing the s *vs.* c dependence of the pure species.

In the light of these considerations let us now examine the potentialities of the difference sedimentation technique (Kirschner and Schachman, 1971a) which can measure very small changes in the weight-average sedimentation coefficient caused by the addition of a ligand. If the concentration range accessible to study is limited to that where s decreases with c for both case I and case II the presence of an association–dissociation equilibrium may not be recognized and the change in s caused by the ligand would be ascribed erroneously to a change in the frictional coefficient instead of a shift in the association–dissociation equilibrium. As will be shown later for systems exhibiting little dissociation the value of the association constant, K, can vary appreciably without a concomitant large change in the s *vs.* c dependence in this concentration region (this change may be so small as to escape detection by the use of conventional methods). These same changes in K have such slight effects on the molecular weight that they may escape detection by the sedimentation equilibrium methods at their present level of sensitivity. Hence a change in s upon the addition of a ligand at one protein concentration would yield equivocal results in terms of distinguishing between molecular weight and shape or volume changes. However, if the slope of the s *vs.* c curve can also be measured, both with and without the ligand, then the change in the concentration dependence of s may be used to estimate the contribution of the shift in the association–dissociation equilibrium to the observed changes in the sedimentation coefficient at a fixed protein concentration. In this calculation it is assumed that the concentration dependence of the sedimentation coefficient is altered only negligibly by slight changes in the shape or volume of the protein molecules. Quantitative data of the precision required to test this assumption are not available, but the factors which affect the concentration dependence are qualitatively understood and it seems likely that small shape changes which alter the frictional coefficient and hence s by 1%, for example, will have only a slight effect on the concentration dependence of s.

In the following sections we first derive equations to relate the magnitude of the expected difference in s at a given concentration to the ligand promoted change in K for a monomer–dimer equilibrium. Then we derive corresponding equations relating K to the concentration dependence of s. Slight changes in the concentration dependence of s may be measured either by performing difference sedimentation experiments at various protein concentrations or by performing differential sedimentation experiments in synthetic boundary cells as described by Hersh and Schachman (1955). The relationship between these two methods is illustrated and their relative merits are discussed. If the value of K calculated from the concentration dependence of s corresponds to that calculated from the observed difference in sedimentation coefficient at a fixed concentration, then one can assume that the change in s is wholly due to a shift in the association–dissociation equilibrium. If the two values differ, the change in s must be due at least in part to a change in the frictional factor of individual polypeptide species.

All calculations are illustrated for a protein[1] of molecular weight 1.44×10^5, and a sedimentation coefficient at infinite dilution of 7.6 S. For simplicity in one case we treat the protein as a dimer which dissociates slightly (and reversibly) to form monomers of mol wt 0.72×10^5. In the other case, to assess the effect of a small amount of association caused by the ligand, we consider the same protein to be a monomer which associates reversibly to a slight extent to form dimers of mol wt 2.88×10^5. Through a consideration of these two models with different equilibrium constants we can assess the effect of a shift in the chemical equilibrium for the hypothetical protein on its sedimentation coefficient and the concentration dependence of the sedimentation coefficient.

Relationship between the Change in Sedimentation Coefficient and a Shift in the Association–Dissociation Equilibrium

If a ligand causes a small amount of association or dissociation of a protein, there will be a slight change in s (Figure 1) which is measurable by the difference sedimentation technique. In order to evaluate the sensitivity of the sedimentation velocity methods to slight changes in the equilibrium between dimers and monomers we assume that there is no shape change in the molecules upon the addition of ligand and that the dependence of s upon c is the same for monomer and dimer. Since for a globular protein species the sedimentation coefficient, s, is generally related to total concentration, c, by the relationship

$$s = s^0(1 - kc) \qquad (1)$$

[1] The parameters chosen for the calculations refer to the enzyme, rabbit muscle glyceraldehyde phosphate dehydrogenase which has a molecular weight of 1.44×10^5 (Harrington and Karr, 1965; Harris and Perham, 1968), a sedimentation coefficient, at infinite dilution, of 7.6 S and a concentration dependence of the sedimentation coefficient with $k = 0.008$ l./g (Hoagland and Teller, 1969; Meighen and Schachman, 1970). The tetrameric enzyme has been shown to dissociate into dimers of mol wt 7.2×10^4 and to aggregate slightly into octamers of mol wt 2.88×10^5 (Hoagland and Teller, 1969). Studies on this enzyme relevant to the theory presented here are described in the preceding paper (Smith and Schachman, 1972).

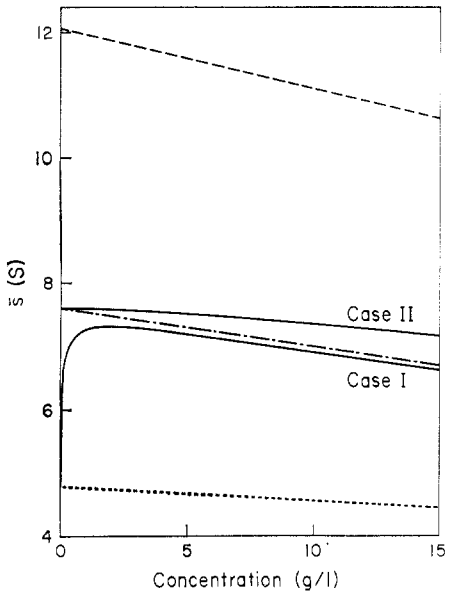

FIGURE 1: The weight-average sedimentation coefficient, $\bar{s}$, is plotted as a function of total concentration (grams per liter) for two systems containing monomer in equilibrium with dimer. In the first case (solid line, case I) a dimeric protein of $s^0 = 7.6$ S is in equilibrium with monomer, the concentration of each being related by an association constant, $K = 100$ l./g. Both the monomer and dimer are considered to have a dependence of sedimentation coefficient, s, on concentration, c, as described by the equation $s = s^0(1 - kc)$, where $k = 0.0080$ l./g. The sedimentation coefficient of the monomer is taken as 0.63 times that of the dimer. The line (- · - · -) represents the $\bar{s}$ *vs.* c dependence of pure dimer and (· · · · ·) represents that of pure monomer. For the second system (solid line, case II) the protein whose $s^0 = 7.6$ S and whose $\bar{s}$ *vs.* c dependence is shown by the line (- · - · -) is considered to be a monomer which is in equilibrium with dimer as defined by the association constant, $K = 0.01$ l./g. Again the s of the monomer is 0.63 that of dimer and both have a $k = 0.0080$ l./g. The $\bar{s}$ *vs.* c dependence of the dimer in this case is denoted by the line (- - -).

where s^0 is the sedimentation coefficient at infinite dilution, our assumption requires that the value of k be the same for both monomer and dimer. For most globular proteins, irrespective of their molecular weight, k varies from 0.006 to 0.009 l. per g (Creeth and Knight, 1965) as long as charge effects are minimized by the presence of dilute salt or the proteins have a small net charge. A rigorous hydrodynamic treatment of this problem is lacking even for a single, rigid macromolecular component. For mixtures of noninteracting components, 1 and 2, it has been shown that two terms, k_1c_1 and k_2c_2, are required to account for the dependence of s upon c (Schachman, 1959). Until precise data become available for model systems simulating monomers and dimers we are constrained to make the approximation that the k values for monomer and dimer are equal. Obviously if there is a large shape change, as in a rodlike dimer dissociating either laterally or longitudinally, the axial ratio would be doubled or halved, respectively, and the value of k for the dimer would not equal that for the monomer. Such cases are not considered here and the treatment is restricted to compact, globular macromolecules for both monomer and dimer.

Effect of a Small Degree of Dissociation. Consider that in a difference sedimentation experiment involving a dimeric protein, D, the observed fractional change in sedimentation coefficient results from a small degree of dissociation of D into monomer, M, of the protein in one side of the centrifuge cell as compared to the pure dimer in the other sector. We assume that the total difference in sedimentation coefficient is due to the dissociation and not to a conformational change in D and that the concentration dependence of the sedimentation coefficient is the same for D as for M. The equilibrium between the monomer and dimer is described by an association constant K in liters per gram

$$K = \frac{c_D}{c_M^2} \tag{2}$$

where c_D is the concentration of dimer and c_M is the concentration of monomer on a weight–volume basis (grams per liter). For this equilibrium mixture the weight-average sedimentation coefficient, $\bar{s}$, can be written

$$\bar{s} = \frac{s_M c_M + s_D c_D}{c} \tag{3}$$

where c is the total concentration and s_M and s_D are the sedimentation coefficients of the monomer and dimer, respectively. The observed fractional difference in sedimentation coefficient between the equilibrium mixture and pure dimer, $\Delta\bar{s}/\bar{s}_{av}$[2], is

$$\frac{\Delta\bar{s}}{\bar{s}_{av}} = \frac{2(\bar{s} - s_D)}{\bar{s} + s_D} \tag{4}$$

Dissociation therefore produces a negative $\Delta\bar{s}/\bar{s}_{av}$. Since c_M and c_D are related by

$$c = c_M + c_D \tag{5}$$

we can obtain a relationship between $\Delta\bar{s}/\bar{s}_{av}$, K, and c by combining eq 2, 3, 4, and 5

$$K = \frac{(2 - \Delta\bar{s}/\bar{s}_{av})(1 - s_M/s_D)[2(1 - s_M/s_D) + \Delta\bar{s}/\bar{s}_{av}(1 + s_M/s_D)]}{4(\Delta\bar{s}/\bar{s}_{av})^2 c} \tag{6}$$

For small values of $\Delta\bar{s}/\bar{s}_{av}$ (less than 5%), eq 6 may be approximated by

$$K = \left[\frac{1 - s_M/s_D}{\Delta\bar{s}/\bar{s}_{av}}\right]^2 \frac{1}{c} \tag{6a}$$

Equation 6a indicates that for the type of system under consideration the smaller the value of K the larger the $\Delta\bar{s}/\bar{s}_{av}$ and that for a given K the absolute value of $\Delta\bar{s}/\bar{s}_{av}$ increases somewhat with decreasing concentration. Therefore, for this type of system, to optimize sensitivity one will have to compromise between the increased accuracy of the difference sedimentation measurements at higher concentration and the somewhat larger $\Delta\bar{s}/\bar{s}_{av}$ expected at lower concentration.

Equation 6 may be employed to calculate $\Delta\bar{s}/\bar{s}_{av}$ as a function of K and c. Table I shows values of $\Delta\bar{s}/\bar{s}_{av}$ calculated for

[2] The term, $\bar{s}_{av}$, refers to the average of the weight-average sedimentation coefficients of the protein in the two solutions being compared. The difference between the two weight-average sedimentation coefficients is designated by $\Delta\bar{s}$. In a previous discussion of this problem (Kirschner and Schachman, 1971a) where associating–dissociating systems were not specifically treated, the fractional difference in sedimentation coefficients was written as $\Delta s/\bar{s}$.

TABLE I: Effect of Association–Dissociation Equilibrium on Sedimentation Coefficient and Its Concentration Dependence.[a]

Case	K (l./g)	Monomer (%)	M_W	$\Delta s/s_{av}$ (%)	k_{app} (l./g)
I	∞	0	144,000	0	0.0080
	1000	1.0	143,283	−0.4	0.0078
	100	3.1	141,759	−1.2	0.0075
	25	6.1	139,590	−2.3	0.0070
	10	9.5	137,152	−3.6	0.0063
	5	13.2	134,513	−5.0	0.0056
		Dimer (%)			
II	0	0	144,000	0	0.0080
	0.0010	1.0	145,411	0.6	0.0075
	0.0025	2.3	147,433	1.4	0.0068
	0.0050	4.5	150,562	2.7	0.0055
	0.0100	8.4	156,083	4.9	0.0031

[a] The calculations refer to a system of monomer in rapid equilibrium with dimer where the association constant (K) is either very large (case I) or very small (case II). The $\Delta s/s_{av}$ values are those expected from eq 6 or 11 when the sedimentation coefficients of such systems are compared with those of pure dimer (case I) or pure monomer (case II), respectively. The k_{app} values were calculated from eq 23 (case I) and 24 (case II) and show how the concentration dependence of the weight-average sedimentation coefficient varies with K for each type of system. Both monomer and dimer are assumed to have $k = 0.0080$ and $s_M/s_D = 0.63$. All calculations refer to a concentration of 10 g/l. and the weight-average molecular weight (M_W) and percent monomer or dimer for each value of K are also shown.

various values of K assuming a total concentration of c = 10 g/l. and an s_M/s_D of 0.63 as predicted for spherical proteins (Svedberg and Pedersen, 1940). For an infinite association constant there is no monomer present and $\Delta s/s_{av}$ is zero. For an association constant of 100 l./g, the weight-average molecular weight, M_w, drops from 144,000 to 141,759 with the production of 3.1% monomer, a change which would be difficult to measure by sedimentation equilibrium measurements at their present level of sophistication (Yphantis, 1964; Roark and Yphantis, 1970; Teller *et al.*, 1970). The corresponding $\Delta s/s_{av}$ of −1.2% can, however, be measured with high precision (Kirschner and Schachman, 1971a). This is more clearly seen in Figure 2, which shows plots of both weight-average molecular weight, M_w, and s as a function of c for the values of K used in Table I. For K values of 1000 and 100, for example, it is seen that in the concentration range for which the high-speed sedimentation equilibrium method is most accurate (0.1–3.0 g/l.) M_w varies little; even if the dissociation were detectable, its extent would be impossible to measure with sufficient accuracy to allow one to conclude that a measured change in s was due wholly to the change in molecular weight. Similarly, as shown in Figure 2B for the highly associated systems, the s *vs.* c curves are linear and have a negative slope in the region accessible to schlieren optics and the presence of the small amount of dissociation would be very difficult to detect from a series of sedimentation velocity experiments. Nevertheless, such dissociation will produce differences in sedimentation coefficient easily measurable with the difference sedimentation method.

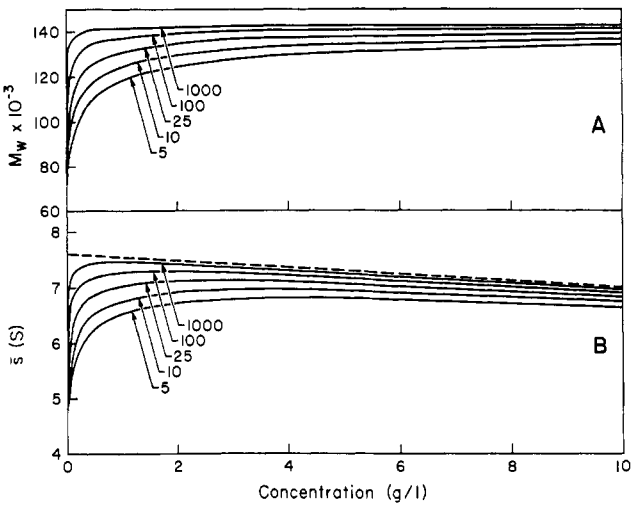

FIGURE 2: Plots of weight-average molecular weight, M_w, in part A and weight-average sedimentation coefficient, $\bar{s}$, in part B against concentration (grams per liter) for systems of monomer in equilibrium with dimer. The association constants (liters per gram) for the various systems are indicated in the figure. The monomer has a molecular weight of 7.2×10^4 and its s value is 0.63 times that of the dimer which has an s^0 of 7.6 S. The dotted line in part B represents the s *vs.* c dependence of pure dimer. Both monomer and dimer have an s *vs.* c dependence which is described by $s = s^0(1 - kc)$ where $k = 0.0080$ l./g.

The change in sedimentation coefficient upon the addition of a ligand could, of course, arise as a result of shift of the association–dissociation equilibrium with dissociation occurring in both solutions. In this case $\Delta s/s_{av}$ can be related to two equilibrium constants, K_1 and K_2, where K_1 and K_2 are the association constants in the absence and presence of the ligand. From eq 2 and 5 c_D is related to c and K by

$$c_D = \frac{2c + 1/K - \sqrt{4c/K + 1/K^2}}{2} \tag{7}$$

Expansion of the quadratic term and neglecting terms involving higher powers of $1/K$ in eq 7 lead to

$$c_D = c - \sqrt{c/K} \tag{8a}$$

and hence from eq 5 we obtain

$$c_M = \sqrt{c/K} \tag{8b}$$

Equations analogous to (3) may be written for the systems described by K_1 and K_2 and upon substitution into them of eq 8a and 8b one can derive

$$\frac{\Delta s}{s_{av}} = \frac{2(1 - s_M/s_D)(1/\sqrt{K_1} - 1/\sqrt{K_2})}{2\sqrt{c} - (1 - s_M/s_D)(1/\sqrt{K_1} + 1/\sqrt{K_2})} \tag{9}$$

For large K, eq 9 can be approximated by

$$\frac{\Delta s}{s_{av}} = \frac{(1 - s_M/s_D)(1/\sqrt{K_1} - 1/\sqrt{K_2})}{\sqrt{c}} \tag{9a}$$

If K_1 or K_2 is equal to infinity, that is, one sample is composed of pure dimer, then eq 9a reduces to eq 6a. Though a

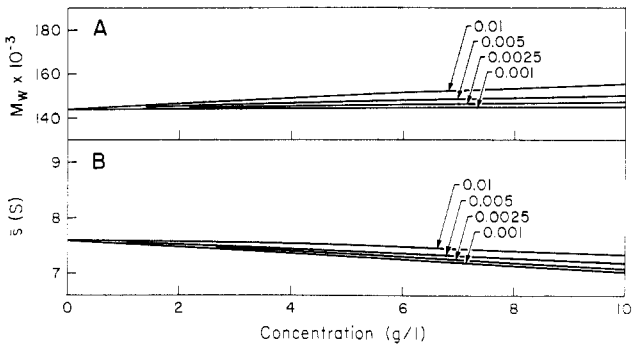

FIGURE 3: Plots of weight-average molecular weight, M_w, in part A and weight-average sedimentation coefficient, $\bar{s}$, in part B against concentration (grams per liter) for systems of monomer in equilibrium with dimer. The association constants (liters per gram) for the various systems are indicated in the figure. The monomer has a molecular weight of 1.44×10^5 and its s^0 value (7.6 S) is 0.63 times that of the dimer. Both monomer and dimer have an s *vs.* c dependence which is described by $s = s^0(1 - kc)$ where k = 0.0080 l./g.

given $(\Delta s/s_{av})$ can represent an infinite number of pairs of K_1 and K_2, from eq 9a it is seen that $\Delta s/s_{av}$ does represent a difference in the degree of dissociation which is proportional to $(1/\sqrt{K_1} - 1/\sqrt{K_2})$.

Effect of a Small Degree of Association. As seen in Figure 3A,B the plots of M_w and s as a function of concentration for systems showing little tendency to associate are essentially linear throughout the concentration range accessible to Rayleigh and schlieren optics. Hence direct measurements of M_w and s may fail to reveal the association–dissociation equilibrium. For such systems we can assume that the binding of ligands converts a monomeric protein into a monomer–dimer equilibrium mixture. Again we assume that the entire difference in sedimentation coefficient is due to the association and not to a change in the frictional factor of the monomers, and that the concentration dependence of the sedimentation coefficient is the same for monomer and dimer. The fractional difference in sedimentation coefficient is given by

$$\frac{\Delta s}{s_{av}} = \frac{2(s - s_M)}{s + s_M} \tag{10}$$

Association therefore produces a positive $\Delta s/s_{av}$. From eq 2, 3, 5, and 10 we derive for the association constant (liters per gram)

$$K = \frac{(2\Delta s/s_{av})(2 - \Delta s/s_{av})s_M/s_D}{(1 - s_M/s_D)[2 - (1 + s_M/s_D)/(1 - s_M/s_D)\Delta s/s_{av}]^2 c} \tag{11}$$

For small $\Delta s/s_{av}$ eq 11 is approximated by

$$K = \frac{\Delta s/s_{av}}{(s_D/s_M - 1)c} \tag{11a}$$

From c and a reasonable value for s_M/s_D (0.63 for spheres), a value of $\Delta s/s_{av}$ can again be calculated for each K. Such calculations, based on eq 11 and a concentration of 10 g/l., are shown in Table I. It is clearly seen from this table that the sedimentation coefficient is very sensitive to small amounts of aggregation. For the system under consideration, increases in sedimentation coefficient of approximately 2% or less could represent an increase in weight-average molecular weight too small to measure by conventional techniques such as sedimentation equilibrium. For this case, as opposed to cases in which ligand binding causes a small degree of dissociation, it is seen from eq 11a that the difference in sedimentation coefficient increases with increasing concentration.

Again, the difference in sedimentation coefficient could arise from a slight degree of association occurring in both solutions. For systems where K is small, *i.e.*, $c \ll 1/K$, the square-root term of eq 7 can be expanded by the binomial theorem and approximated by

$$c_D = Kc^2 \tag{12a}$$

Hence, from eq 5

$$c_M = c - Kc^2 \tag{12b}$$

Using these approximations in the form of eq 12a and 12b we can derive an equation analogous to eq 9 which relates $\Delta s/s_{av}$ to K_1 and K_2, the association constants describing the two systems in the absence and presence of the ligands. This takes the form

$$\frac{\Delta s}{s_{av}} = \frac{2(K_2 - K_1)(1 - s_M/s_D)c}{2s_M/s_D + (K_1 + K_2)(1 + s_M/s_D)c} \tag{13}$$

For small values of K eq 13 simplifies to

$$\Delta s/s_{av} = (K_2 - K_1)(s_D/s_M - 1)c \tag{13a}$$

If $K_1 = 0$, eq 13a becomes eq 11a. At any given c it is seen from eq 13a that the difference in sedimentation coefficient is approximately proportional to the difference in the equilibrium constants.

Since in general it is possible that ligand binding alters the frictional factor of the protein in addition to altering its state of association, it would be hazardous to use measurements of $\Delta s/s_{av}$ at a single protein concentration to determine K for systems suspected of showing either a small degree of association or dissociation. However, as shown in the next section, measurements of $\Delta s/s_{av}$ at a series of concentrations, or determination of the change in the concentration dependence of the sedimentation coefficient, can be used to determine approximately the value of K by a method which is much less sensitive to possible changes in the frictional factor.

Effect of Association–Dissociation Equilibrium on the Concentration Dependence of the Sedimentation Coefficient

For globular noninteracting proteins s is related to c by eq 1 which illustrates the linear relationship between the two parameters. From eq 1 it follows that k can be defined by

$$k = -\frac{ds/dc}{s^0} \tag{14}$$

Combining eq 1 and 14 leads to the elimination of s^0 and gives

$$k = -\frac{d \ln s/dc}{1 - (d \ln s/dc)c} \tag{15}$$

From eq 15 the value of k can be determined from the dependence of s on c without requiring extrapolation of the data to infinite dilution to obtain s^0. For associating systems such as those described by cases I and II in Figure 1, in the concentration range where the s *vs.* c dependence is negative, it becomes useful to define a parameter, k_{app}, by analogy with eq 15. Thus we can write

$$k_{app} = -\frac{d \ln s/dc}{1 - (d \ln s/dc)c} \tag{15a}$$

The value of k_{app}, which is determined from the dependence of s upon c, is less than that of k for the noninteracting protein because of the positive contribution resulting from self-association.

In practice differential sedimentation experiments with the synthetic boundary cell give $\Delta s/\Delta c$ directly from the difference in the rate of movement of the differential boundary, s_{diff}, and the sedimentation coefficient, s_1, of the upper solution which is at a concentration, c_1. The sedimentation coefficient in the lower solution is s_2 at concentration, c_2. For small concentration differences we can write (Hersh and Schachman, 1955)[3]

$$s_{diff} - s_1 = c_2\frac{ds}{dc} \tag{16}$$

From this expression we have

$$\frac{d \ln s}{dc} = [(s_{diff}/s_1) - 1]/c_2 \tag{17}$$

Equation 15a may then be written in terms evaluated directly from differential sedimentation experiments

$$k = \frac{[(s_1/s_{diff}) - 1]/c_2}{2 - (s_1/s_{diff})} \tag{18}$$

When the difference in concentration is appreciable, then eq 17 is an approximation. The differential, $(\Delta \ln s)/\Delta c$, may be written as

$$\frac{\Delta \ln s}{\Delta c} = \frac{1}{c_2}\left[\frac{2(s_{diff} - s_1)}{s_1 + s_2} - \frac{1}{2}\left(\frac{2(s_{diff} - s_1)}{s_1 + s_2}\right)^2 + \frac{1}{3}\left(\frac{2(s_{diff} - s_1)}{s_1 + s_2}\right)^3 - \cdots\right] \tag{19}$$

where s_2 can be determined in a differential sedimentation experiment from s_1 and s_{diff} by

$$s_2 = s_{diff} - \frac{c_1}{c_2}(s_{diff} - s_1) \tag{20}$$

For most experiments the higher terms in eq 19 can be neglected and an equation analogous to (18) may be used to compute k_{app} from differential sedimentation experiments

$$k_{app} = \frac{\left[\frac{2(s_1 - s_{diff})}{s_1 + s_2}\right]\frac{1}{c_2}}{1 + \left[\frac{2(s_1 - s_{diff})}{s_1 + s_2}\right]} \tag{21}$$

The concentration at which k_{app} is then determined is the average concentration, $(c_1 + c_2)/2$.

We now consider a monomer–dimer system for which the equilibrium constant is described by eq 2. If both M and D have the same dependence of sedimentation coefficient on c (*i.e.*, same k) then s_M and s_D may be related to c by expressions analogous to eq 1. On substituting these expressions into eq 3 one obtains an expression for the weight-average sedimentation coefficient of the mixture over a range of concentrations

$$s = s_M^0(1 - kc)c_M/c + s_D^0(1 - kc)c_D/c \tag{22}$$

where s_M^0 and s_D^0 are the sedimentation coefficients of M and D at infinite dilution.

Effect of a Small Degree of Dissociation on k_{app}. For systems in which K is large (*e.g.*, case I, Figure 1) c_M and c_D can be approximated by eq 8a and 8b. Upon taking the logarithm of eq 22, combining it with eq 8a and 8b, differentiating and substituting into eq 15a one obtains

$$k_{app} = \frac{2kK^{1/2}c^{3/2} - (1 + kc)(1 - s_M/s_D)}{2K^{1/2}c^{3/2} + c(kc - 3)(1 - s_M/s_D)} \tag{23}$$

For the type of system under consideration (high K) eq 23 may be approximated to give

$$k_{app} = k - \frac{(1 + kc)(1 - s_M/s_D)}{2K^{1/2}c^{3/2}} \tag{23a}$$

Equations 23 and 23a show that the k_{app} value observed for a monomer–dimer system of high K is lower than the value expected for pure dimer by an amount which is related inversely to the square root of the equilibrium constant and to $c^{3/2}$. In Table I, values of k_{app} at 10 g/l. are calculated from eq 23 for those values of K for which $\Delta s/s_{av}$ were calculated in the last section. Also in Table II, eq 23a has been employed to calculate k_{app} as a function of concentration for the same values of K. In both cases k has been taken as 0.008 l./g and s_M/s_D as 0.63. Two points may be made from these calculations. First, at a fixed concentration the parameter k_{app} is very sensitive to changes in K. If a dimer were to exist in rapid equilibrium with a small amount of monomer as described by the equilibrium constant 100 l./g, k_{app} at 10 g/l. would be 0.0075 l./g as compared with pure dimer of k_{app} = 0.0080 l./g. Since values of k_{app} can be determined by the technique of Hersh and Schachman (1955) to approximately 0.0003 such a change should be readily detectable. Such a change would correspond to a 1.2% change in s and to a change of M_w of only 144,000 to 141,759 (Table I). Second, as seen in Table II, k_{app} is very sensitive to changes in c. For K = 100 l./g, decreasing the concentration from 10 to 5 g per l. decreases k_{app} from 0.0075 to 0.0064 l. per gram, a difference easily observed. Figure 2 shows that the corresponding molecular weight changes would not be so readily detected.

[3] In their formulation Hersh and Schachman (1955) considered a finite concentration difference between the upper and lower solutions and the appropriate equation describing s_{diff} contained $\Delta s/\Delta c$ which was equal to $(s_2 - s_1)/(c_2 - c_1)$. When the concentration difference is small we can write ds/dc for $\Delta s/\Delta c$ especially since, for noninteracting systems, s varies almost linearly with concentration. The term c_2 in eq 16 could, of course, be written as c. For small concentration differences s_1 is very close to s_2 and we can write $(1/s_1)ds/dc$ as $(d \ln s)/dc$.

TABLE II: Effect of Variations in Association–Dissociation Equilibrium on Concentration Dependence of Sedimentation Coefficient.[a]

Case	K (l./g)	k_{app} (l./g) 2 g/l.	5 g/l.	10 g/l.
I	∞	0.0080	0.0080	0.0080
	1000	0.0060	0.0075	0.0078
	100	0.0014	0.0064	0.0075
	25	−0.0057	0.0048	0.0070
	10	−0.0148	0.0028	0.0063
	5	−0.0263	0.0003	0.0056
II	0	0.0080	0.0080	0.0080
	0.001	0.0074	0.0075	0.0075
	0.0025	0.0066	0.0067	0.0068
	0.005	0.0052	0.0053	0.0055
	0.010	0.0023	0.0026	0.0031

[a] Values for k_{app} in liters per gram are tabulated at three different concentrations and for different values of the association constant K in liters per gram for a system of monomer in rapid equilibrium with dimer. Two cases are considered: case I where K is large and case II where K is small. The values of k_{app} were calculated from eq 23 and 24 for cases I and II, respectively, assuming a k for monomer and dimer of 0.0080 and $s_M/s_D = 0.63$.

Effect of a Small Degree of Association on k_{app}. Again we consider a system described by eq 1 but in which K is very small. Combining eq 22 with eq 12a and 12b, differentiating the logarithm and substituting into eq 15a give

$$k_{app} = \frac{k - (s_D/s_M - 1)(1 - 2kc)K}{1 + kKc(s_D/s_M - 1)} \quad (24)$$

For small k, the term $kKc(s_D/s_M - 1) \ll 1$ and hence eq 24 can be further simplified to give

$$k_{app} = k - (s_D/s_M - 1)(1 - 2kc)K \quad (24a)$$

Equations 24 and 24a demonstrate that for systems showing a slight degree of association k_{app} is lower than the k value expected for pure monomer by a quantity which is proportional to both K and c, in contrast to systems in which the protein is essentially all in the dimer form, in which case k_{app} varies inversely with $K^{1/2}$ and $c^{3/2}$. This conclusion has two implications which are clearly seen in Table II and which would prove useful in distinguishing whether a protein undergoes a small degree of association or dissociation. First, k_{app} for case I (Figure 1) systems is much more sensitive to changes in concentration than that for case II systems. This is seen by examination of Table II. If, for example, binding of ligands to dimer caused it to exist in equilibrium with monomers with an association constant of 100 l./g then k_{app} would drop from 0.0080 to 0.0014 l. per g at 2 g/l. and from 0.0080 to 0.0075 l. per g at 10 g/l. In contrast if this same protein were a monomer and were to associate to dimers with a K of 0.0025 l./g, then k_{app} would vary only from 0.0066 l./g at 2 g/l. to 0.0068 l./g at 10 g/l. Both situations correspond to a $\Delta s/\bar{s}_{av}$ in the range 1.2–1.4% (Table I). Second, k_{app} is much more sensitive to changes in K for case II systems than for case I systems. This is seen from the values in Table I and from the plots of $\bar{s}$ *vs.* c in Figure 3 in comparison to those in Figure 2. As seen in Figure 3 there is only a small change in molecular weight[4] due to association (k = 0.01 l./g) at 2 g/l. However, the value of k_{app} is markedly decreased even with this slight amount of association.

Relationship of Difference Sedimentation Studies as a Function of Concentration to Differential Sedimentation Studies

In experiments of ligand-induced changes in sedimentation coefficient the contribution of an association–dissociation equilibrium of the protein molecules to the difference in s can be detected readily by comparing the concentration dependence of the sedimentation coefficient of the protein in the presence and absence of ligands. The difference in the concentration dependence, Δk_{app}, is a sensitive measure of association or dissociation and is related directly to the difference in the equilibrium constants as shown by eq 23 and 24. The value of Δk_{app} can be determined in a single experiment by simultaneous differential sedimentation experiments using two layering cells (Kirschner and Schachman, 1971b; Smith and Schachman, 1973). For some applications, however, it may be preferable to use the difference sedimentation technique, which is inherently more sensitive, to determine Δk_{app} by measuring the concentration dependence of $\Delta\bar{s}/\bar{s}_{av}$, or $(d/dc)(\Delta\bar{s}/\bar{s}_{av})$. As shown below, the results from the latter type of measurements give values which are simply related to the difference in the concentration dependence, Δk_{app}, and, therefore, on theoretical grounds these two techniques can be used interchangeably. The practical considerations dictating which technique should be employed are discussed below.

In a difference sedimentation experiment, if the two solutions differ in the dependence of their sedimentation coefficient on concentration, then $\Delta\bar{s}/\bar{s}_{av}$ will show a concentration dependence. From the definition of $\Delta\bar{s}/\bar{s}_{av}$ for two homogeneous substances of different k, it can be shown that

$$\frac{\Delta\bar{s}}{\bar{s}_{av}} = \frac{\Delta s^0(1 - \bar{k}c) - \bar{s}^0_{av}\Delta kc}{\bar{s}^0_{av}(1 - \bar{k}c) + \Delta s^0\Delta kc/4} \quad (25)$$

where Δs^0 and $\bar{s}^0_{av}$ are the difference between and the average of the sedimentation coefficients at infinite dilution and $\bar{k}$ and Δk are the average k and the difference between the k's for the two solutions, respectively.

Neglecting the term $\Delta s^0\Delta kc/4$ and rearranging eq 25, we have for the concentration dependence of $\Delta\bar{s}/\bar{s}_{av}$

$$\frac{\Delta\bar{s}}{\bar{s}_{av}} = \frac{\Delta s^0}{\bar{s}^0_{av}} - \frac{\Delta kc}{1 - kc} \quad (26)$$

If $\Delta k = 0$, $\Delta\bar{s}/\bar{s}_{av}$ is independent of concentration. Differentiating eq 26 with respect to concentration gives

$$\frac{d}{dc}\frac{\Delta\bar{s}}{\bar{s}_{av}} = -\frac{\Delta k}{(1 - \bar{k}c)^2} \quad (27)$$

[4] These plots of molecular weight *vs.* concentration were calculated on the assumption that the solutions were ideal; hence the variation in M_w with concentration is attributable only to the change in the relative amounts of monomer and dimer as the total concentration varies. For real systems the apparent molecular weight decreases with increasing concentration as a result of nonideality. The inclusion of appropriate virial coefficients in the calculation of the theoretical curves would affect the magnitude of the values of M_w slightly. However the slopes of the corrected curves of M_w *vs.* c (especially at higher concentrations) would be seriously influenced by changes in K. In that regard the curves of M_w *vs.* c would resemble those of $\bar{s}$ *vs.* c.

Thus, according to eq 27, the value of Δk is obtained directly from the concentration dependence of $\Delta\bar{s}/\bar{s}_{av}$ multiplied by $-(1 - \bar{k}c)^2$. It should be noted, however, that Δk is also obtained directly from the differential sedimentation experiments according to $\Delta[-(1/s^0)ds/dc]$. For self-associating systems eq 27 can be written as

$$\Delta k_{app} = -(1 - \bar{k}_{app}c)^2 \frac{d}{dc}(\Delta\bar{s}/\bar{s}_{av}) \quad (28)$$

Thus data obtained by measuring $\Delta\bar{s}/\bar{s}_{av}$ as a function of concentration can be multiplied by $-(1 - \bar{k}_{app}c)^2$ to give Δk_{app}, and an estimate of the amount of association or dissociation can be made using eq 23 or 24. A reliable value of Δk_{app} can be obtained from eq 28 and measurement of $(d/dc)(\Delta\bar{s}/\bar{s}_{av})$ even without an accurate estimate of $\bar{k}_{app}$.

Discussion

The sedimentation coefficient of a macromolecule is related to both its molecular weight and its frictional coefficient; hence treatments which lead to a substantial alteration of the sedimentation coefficient cannot be interpreted in an unambiguous manner. In some instances, and especially with the availability of other data, it is possible to distinguish between changes in molecular weight or frictional coefficient. With the development of the difference sedimentation technique (Kirschner and Schachman, 1971a), which has the precision for detecting extremely small alterations in sedimentation coefficient, the problem of distinguishing very *small* changes in molecular weight from changes in frictional coefficient has become of considerable significance. This dilemma is particularly acute for associating–dissociating systems for which the equilibrium can be shifted by the addition of specific ligands. In this regard the treatment presented here makes two principal points. First, equations are presented which relate the observed change in sedimentation coefficient to the equilibrium constant and protein concentration for rapidly equilibrating monomer–dimer systems where the association constant is either very large or very small. Second, although difference sedimentation experiments at a single protein concentration do not permit a determination of the relative contributions of a change in molecular weight *vs.* a change in frictional coefficient to the observed alteration in sedimentation coefficient, measurements over a range of protein concentrations do justify conclusions about the magnitude of the change in the association constant.

The theoretical treatment presented here could, of course, be extended to cover systems other than those containing monomer and dimer and also to systems where monomer and dimer are *both* present in considerable amounts. For the latter type of system the presence of an association–dissociation equilibrium will usually be apparent from sedimentation equilibrium experiments; ligand-induced changes in association constant would be recognized and the difference in sedimentation coefficient could be attributed to a shift in the equilibrium. One of our principal aims in this communication, however, has been to draw attention to the fact that oligomeric enzymes may exist in equilibrium with such small amounts of subunits or aggregates that the very presence of the association–dissociation equilibrium, and hence changes thereof, might go undetected. A number of proteins exist which are known to contain noncovalently linked subunits but for which a variety of techniques has failed to reveal the presence of such an equilibrium (Reithel, 1963; Klotz *et al.*, 1970). Hence it is possible that workers who claim to have measured changes in the frictional factor have in fact measured changes in weight-average molecular weight.

In the calculations illustrated by Table I it was assumed that the change in s was due solely to a change in the association constant, K. Even in the absence of a shift in the association–dissociation equilibrium the addition of a ligand could lead to a change in $\bar{s}$ and a much smaller change in k_{app} if the frictional coefficient of the protein were altered due to a change in the shape or volume of the molecules. In that case, calculations of K from k_{app} would yield results which differed from the value of K determined by measurement of $\Delta\bar{s}/\bar{s}_{av}$ at a single concentration. Thus it is important that measurements of both $\Delta\bar{s}/\bar{s}_{av}$ and k_{app} be accurate. Changes in the latter parameter to give Δk_{app} can be obtained either by performing difference sedimentation experiments over a range of concentrations or by performing differential sedimentation experiments in which protein at one concentration is layered over another solution containing protein at a higher concentration (Hersh and Schachman, 1955). The major advantage of the difference sedimentation technique is its inherent precision. A second advantage is that it provides values of Δk_{app} from measurements made over very small concentration ranges. For differential sedimentation experiments the range of concentration over which k_{app} can be measured is limited by the requirement that the two solutions in the cell be of sufficiently different density to provide a stable differential boundary. An advantage of the differential sedimentation technique is that by using two centrifuge cells simultaneously (*e.g.*, Figure 7, previous paper) one can determine Δk_{app} between two samples within a single experiment.[5] This obviates those errors which contribute to the usual scatter in sedimentation velocity data, such as slight uncertainties in temperature or rotor speed, and to which k_{app} is susceptible by virtue of the fact that it contains both a differential and an absolute term (*cf.* eq 14). This differential technique has been used to determine k_{app} for the catalytic subunit of aspartate transcarbamylase (Kirschner and Schachman, 1971b) and for glyceraldehyde phosphate dehydrogenase (Smith and Schachman, 1973). In these two instances the differential boundaries were symmetrical and calculations of s_{diff} were based on the movement of the maximum ordinate of the concentration gradient curve rather than the position corresponding to the second moment of the gradient curve. For skewed boundaries the use of the maximum ordinate would be inappropriate and Δk_{app} should be determined from the concentration dependence of $\Delta\bar{s}/\bar{s}_{av}$. Calculations with the difference sedimentation technique are based on mass transport equations and yield differences in the weight-average sedimentation coefficients directly without assumptions about the shape of the moving boundaries. Thus this method would be preferable for interacting systems in which there are association–dissociation equilibria.

Although the technique of sedimentation equilibrium has obvious theoretical advantages over sedimentation velocity, changes in K can be measured only by performing *separate* experiments of the system under the different conditions. Since the precision in estimating a molecular weight by the high-speed sedimentation equilibrium can be taken as 2–3% at this stage, it is clear that many changes in K of the magnitude being considered would be impossible to detect. As seen from

[5] Schumaker and Adams (1968) previously used this approach for the determination of k by measuring simultaneously the sedimentation coefficients of solutions at two different concentrations.

Tables I and II, however, small changes in K can result in $\Delta s/s_{av}$ values and changes in k_{app} which are readily measurable. Although difference sedimentation equilibrium techniques for measuring small molecular weight change have been proposed for both Rayleigh (Kirschner, 1971; Springer *et al.*, 1972) and absorption (Barlow *et al.*, 1969) optical systems, these techniques as yet are not of sufficient experimental accuracy for the measurements required. Sedimentation velocity experiments are advantageous for labile systems and for substances of high molecular weight where sedimentation equilibrium studies may require experiments of long duration or extremely slow rotor speeds.

A crucial assumption in the treatment presented here is that the changes in the sedimentation coefficient and its concentration dependence are attributable to a shift in the association–dissociation equilibrium and not to alterations in the frictional coefficient. Interpreting either measurement ($\Delta s/s_{av}$ or Δk_{app}) alone would be hazardous since changes in the shape or volume of the hydrodynamic unit would affect each parameter. If, however, measurements of both $\Delta s/s_{av}$ and k_{app} led to similar values of K it would be reasonable to assume that the observed changes were due principally to a shift in the association–dissociation equilibrium. This assumption is justifiable since a change in the frictional coefficient of a globular macromolecule will result in disproportionate changes in the sedimentation coefficient as compared to its concentration dependence (Creeth and Knight, 1965). As yet there has been no complete theoretical treatment of the hydrodynamic factors contributing to the concentration dependence of the sedimentation coefficient (Kermack *et al.*, 1929; Burgers, 1941, 1942; Wales and van Holde, 1954; Schachman, 1959). Nonetheless, empirically it has been found for globular proteins that k is roughly proportional to the intrinsic viscosity with a proportionality constant of about 1.6 (Creeth and Knight, 1965). On this basis an increase of 0.6% in s, if it represented a contraction in the volume of the macromolecules, would be accompanied by approximately a 2% decrease in the intrinsic viscosity and therefore a 3% decrease in k. In the case of the catalytic subunit of aspartate transcarbamylase, for example, there was an increase in s of about 1% upon the addition of ligands and a decrease in k_{app} of 3 ± 2% (Kirschner and Schachman, 1971b). These two changes are consistent with one another and can be interpreted in terms of a reduction in the frictional coefficient of about 1%; they cannot be accounted for if we postulated that the addition of ligands caused a shift in a putative monomer–dimer equilibrium. Such a shift in the equilibrium toward dimer (case II system) would lead to two different values of K if the association constants were calculated independently from the magnitudes of $\Delta s/s_{av}$ and Δk_{app}.

In the case of aspartate transcarbamylase the changes in s and k_{app} upon the addition of ligands are clearly incompatible with any interpretation based on an association–dissociation equilibrium. For the intact enzyme $\Delta s/s_{av}$ was −3.5% which, by itself, could be interpreted either as an increase in the frictional coefficient due, for example, to swelling of the protein molecules or as a shift in a reversible equilibrium to smaller species (case I system). The latter alternative would require that the value of k_{app} should decrease upon the addition of the ligands (Table I). It was found, however, that the opposite occurred; k_{app} increased upon the addition of ligands (Gerhart and Schachman, 1968). Thus the results can be interpreted only in terms of an increase in the frictional coefficient. These two examples serve to illustrate how changes in frictional coefficient can be distinguished from shifts in a monomer–dimer equilibrium by their effects on the sedimentation coefficient and its concentration dependence.

In the treatment presented above it was assumed that the various oligomers (monomers and dimers) are essentially spherical and that the s of monomer is 0.63 times that of dimer (Svedberg and Pedersen, 1940). This is a reasonable assumption for most globular proteins. Even if the spheres formed cylinders (or dumbbell-shaped objects) on dimerization the ratio of s_M/s_D would still be 0.66. This range of variation in s_M/s_D would produce an uncertainty of less than 20% in K for a system of dimer in equilibrium with a small amount of monomer and less than 10% for a system of monomer in equilibrium with a small amount of dimer. The theoretical treatment also contains the assumption that the k values for monomer and dimer are equal. Although this approximation has not been verified for any experimental system in which pure monomers and dimers have been examined separately, it is likely that the values of k will vary only slightly. Even if k for the monomer and dimer differed by 20% the effect on K would be less than 1% for a system where K is small. It is of interest that aspartate transcarbamylase of mol wt 3.1×10^5, and both its catalytic subunit, a trimer of mol wt 1×10^5, and regulatory subunit, a dimer of mol wt 3.4×10^4, all have the same k of 0.009 l./g; for this system, at least, k is insensitive to the state of aggregation (Gerhart and Schachman, 1965; Kirschner and Schachman, 1971b; Cohlberg *et al.*, 1972).

A number of specific conclusions can be drawn with regard to the results on various system. If k_{app} is found to be markedly concentration dependent the protein is likely to consist predominantly of associated species with a small amount of dissociation as seen in Table II for case I systems. Other differences between the two types of systems are seen in Table I and Figures 2 and 3. If the protein were in equilibrium with small amounts of subunits the absolute value of $\Delta s/s_{av}$ would increase with decreasing concentration. In contrast, for a protein which tended to aggregate slightly in a reversible manner $\Delta s/s_{av}$ would increase with increasing concentration. Since the values of k_{app} vary so much with concentration (and even change sign) for case I systems, shifts in the equilibrium upon the addition of ligands would be detected readily by measurements of Δk_{app}. Despite the uncertainty in the value of k for a pure species (associated form) it would be satisfactory to estimate the value of k since Δk_{app} is not sensitive to small variations in the value of k used for the computations. Obviously the utility of the treatment presented here would be enhanced substantially if reliable values of k_{app} were obtained for many different proteins. When one considers the inherent errors in this determination from a number of separate sedimentation velocity experiments at different concentrations (in order to construct an s *vs.* c plot), it is clear that a small degree of association or dissociation might go undetected. Therefore we wish to stress the need for more accurate determinations of the concentration dependence of the sedimentation coefficient of globular proteins.

References

Barlow, G. H., Summaria, L., and Robbins, K. C. (1969), *J. Biol. Chem. 244*, 1138.

Burgers, J. M. (1941), *Proc. Acad. Sci. Amsterdam 44*, 1045, 1177.

Burgers, J. M. (1942), *Proc. Acad. Sci. Amsterdam 45*, 9, 126.

Cohlberg, J. A., Pigiet, V. P., and Schachman, H. K. (1972), *Biochemistry 11*, 3396.

Creeth, J. M., and Knight, C. G. (1965), *Biochim. Biophys. Acta 102*, 549.
Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.
Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538.
Gilbert, G. A. (1955), *Discuss. Faraday Soc., No. 20*, 68.
Gilbert, G. A. (1960), *Nature (London) 186*, 882.
Gilbert, L. M., and Gilbert, G. A. (1961), *Nature (London) 192*, 1181.
Gilbert, L. M., and Gilbert, G. A. (1962), *Nature (London) 194*, 1173.
Goers, J. W., and Schumaker, V. N. (1970), *J. Mol. Biol. 54*, 125.
Harrington, W. F., and Karr, G. M. (1965), *J. Mol. Biol. 13*, 885.
Harris, J. I., and Perham, R. N. (1968), *Nature (London) 219*, 1025.
Hersh, R., and Schachman, H. K. (1955), *J. Amer. Chem. Soc. 77*, 5228.
Hoagland, V. D., Jr., and Teller, D. C. (1969), *Biochemistry 8*, 594.
Kermack, W. O., McKendrick, A. G., and Ponder, E. (1929), *Proc. Roy. Soc. Edinburgh, Sect. B 49*, 170.
Kirschner, M. W. (1971), Ph.D. Thesis, University of California, Berkeley, Calif.
Kirschner, M. W., and Schachman, H. K. (1971a), *Biochemistry 10*, 1900.
Kirschner, M. W., and Schachman, H. K. (1971b), *Biochemistry 10*, 1919.
Klotz, I. M., Langerman, N. R., and Darnall, D. W. (1970), *Annu. Rev. Biochem. 39*, 25.
Meighen, E. A., and Schachman, H. K. (1970), *Biochemistry 9*, 1177.
Reithel, F. J. (1963), *Advan. Protein Chem. 18*, 124.
Richards, E. G., and Schachman, H. K. (1957), *J. Amer. Chem. Soc. 79*, 5324.
Roark, D. E., and Yphantis, D. A. (1970), *Ann. N. Y. Acad. Sci. 164*, 245.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.
Schumaker, V., and Adams, P. (1968), *Biochemistry 7*, 3422.
Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.
Smith, G. D., and Schachman, H. K. (1973), *Biochemistry 12*, 3789.
Springer, M., Kirschner, M., and Schachman, H. K. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, Abstr. 1434.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University Press.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1970), *Ann. N. Y. Acad. Sci. 164*, 66.
Wales, M., and van Holde, K. E. (1954), *J. Polym. Sci. 14*, 81.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Proton Magnetic Resonance Assignments of the Polypeptide Antibiotic Telomycin†

N. G. Kumar and D. W. Urry*

ABSTRACT: The 220-MHz proton magnetic resonance spectrum of telomycin, a cyclic undecapeptide antibiotic, has been analyzed, and the resonances have been assigned to specific hydrogens of the constituent amino acids. The majority of resonance assignments were made possible by proton homonuclear spin-decoupling experiments in different solvents at different temperatures. Temperature dependence of the peptide NH chemical shifts in different solvents was also utilized in confirming the identification of amide resonances and in achieving complete assignment for a given solvent and temperature.

Telomycin, an undecapeptide antibiotic, was isolated in 1958 by Misiek *et al.* from the culture broth of an unidentified streptomyces. Sheehan *et al.* (1963, 1968) determined the primary structure of telomycin as given in Figure 1. Hle is *erythro*-3-hydroxyleucine[1] and Δ-Trp is α,β-didehydrotryptophan. *trans*-3-Hyp and *cis*-3-Hyp are *trans*- and *cis*-3-hydroxyproline, respectively. All amino acids in telomycin are reported to be of the L configuration (Sheehan *et al.*, 1963, 1968).

One prerequisite for a successful conformational analysis of a polypeptide by nmr is the identification of the resonance pattern of individual protons of the constituent amino acids and assignment of individual resonances to specific protons. Therefore, in this paper we wish to report the analysis of the nmr spectra of telomycin in dimethyl-d_6 sulfoxide and the assignment of proton resonances to specific hydrogen atoms. In dimethyl-d_6 sulfoxide, telomycin exhibited features indicative of defined secondary structure. These conformational aspects will be discussed in a subsequent paper.

Experimental Section

Spectra were recorded on a Varian Associates HR-220 spectrometer. Chemical shifts were measured relative to tetramethylsilane as an internal reference. The chemical-shift

† From the Laboratory of Molecular Biophysics, the University of Alabama Medical Center, Birmingham, Alabama 35294. *Received March 9, 1973.*

[1] Abbreviations used are: Hle, *erythro*-3-hydroxyleucine; Δ-Trp, α,β-didehydrotryptophan; 3-Hyp, 3-hydroxyproline; nmr, nuclear magnetic resonance; BAWP, butanol–glacial acetic acid–water–propanol (30:6:24:20); α-L-Thr, *allo*-L-threonine.