# An Associating Low-Sulfur Protein from Wool*

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ABSTRACT: The preceding paper described measurements of the molecular weight of the low-sulfur protein component 8 from wool in solutions containing 8 M urea where the molecular weight was found to be 46,000. In this paper a study of the molecular weight of component 8 in 0.01 M sodium borate is reported. It is found that component 8 is an associating system under such conditions and the molecular weight of the smallest unit present is 23,000. The method developed by Adams and Filmer (Adams, E. T., Jr. (1965), *Biochemistry 4*, 1646; Adams, E. T. Jr., and Filmer, D. L. (1966), *Biochemistry 5*, 2971) for analyzing sedimentation equilibrium data from a nonideal associating protein system was successful in describing the experimental results in terms of equilibrium constants and a virial coefficient. It is concluded that the system is probably an equilibrium between monomer, dimer, and trimer with the trimer as the polymer in much the greater proportion.

The studies of the molecular weight of the reduced and alkylated low-sulfur protein termed component 8 which were described in the preceding paper were made in solutions containing 8 M urea. In this paper the results of some sedimentation equilibrium experiments with component 8 in the absence of urea are reported. Previous studies on the mixed low-sulfur fraction (SCMKA)[1] from wool in solutions not containing urea have been performed at moderate salt concentrations (O'Donnell and Woods, 1956; Harrap and Woods, 1958a,b). There is evidence that the presence of salt favors aggregation of SCMKA in the absence of urea (O'Donnell and Woods, 1956), and it was thought that an examination of the aggregation behavior of component 8 at low salt concentration would be of interest in relation to its behavior in 8 M urea.

The two samples of component 8, sample A and sample S, referred to in the previous paper have also been used in this work. Most of the experiments were done with sample S because apparently it consists of 95% or more of a protein of a single molecular weight. LS and MD (Yphantis, 1964) sedimentation equilibrium experiments were performed with both samples under one set of conditions of pH, ionic strength, and temperature. Some experiments with mixtures of β-lactoglobulin and ovalbumin and ribonuclease and ovalbumin performed to test the sensitivity of the meniscus depletion technique to heterogeneity are also reported.

## Materials

*Buffer.* The buffer salt was analytical reagent grade sodium tetraborate and 0.01 M solutions were made with glass-distilled water. Sodium borate is a pH standard and the pH of 0.01 M solutions was very close to pH 9.2 as prepared; where necessary, however, the pH was adjusted to 9.20 by the addition of a few drops of concentrated sodium hydroxide solution.

*Proteins.* β-Lactoglobulin (Pentex crystalline lot 4814), ovalbumin (Pentex five-times-crystallized lot 5), and ribonuclease (Sigma salt and protease-free lot R100B-69) solutions were prepared by dissolving the solid protein in sufficient buffer to give a concentration of about 1 g/100 ml and dialyzing overnight in the cold against 200 times the volume of buffer, on a rocking dialyzer.

The preparation of samples A and S of component 8 was as described in the preceding paper (Jeffrey, 1968). The samples of component 8 were stored in the cold in solutions containing 8 M urea and were freed of urea by dialysis on a rocking dialyzer in the cold against three changes, each of volume 1000 times that of the protein solution, of 0.01 M sodium borate buffer (pH 9.20) just before use.

## Methods

*Concentration and Partial Specific Volume.* A value of 0.715 ml/g was used for the partial specific volume of component 8. This was obtained by DeDeurwaerder and Harrap (1964) for SCMKA in aqueous phosphate buffer (0.05 M, pH 6.9) and was measured in accordance with the Casassa–Eisenberg (Casassa and Eisenberg, 1964) definition of macromolecular component, *i.e.*, the quantity measured was $\phi^*$. The present experiments were carried out in 0.01 M sodium borate of pH 9.2 and it is necessary therefore to examine the possible effect of the change of pH and ionic strength on the value of the partial specific volume. Unfortunately, insufficient purified protein is available to allow this to be done experimentally, but a good idea of the magnitude of a possible change can be obtained in the following way. The membrane distribution parameter, Γ, is given by $\Gamma = (i/2)(1 - (iZm_2/4m'_3) + \ldots)$ (Casassa and Eisenberg, 1960), where $Z$ is the charge on the macro-

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: SCMKA, *S*-carboxymethylkerateine A. LS denotes low-speed sedimentation equilibrium experiments, MD meniscus depletion (high-speed) experiments.

molecular species, $m_2$ its molal concentration, $m'_3$ is the concentration of diffusible ions outside the membrane at dialysis equilibrium, and $i$ is a factor by which the stoichiometric charge $Z$ is apparently altered by interactions of unspecified character.

For the Casassa and Eisenberg definition to be applied the numbers of moles of positive and negative ions to be included in 1 mole of electrically neutral macromolecular component are given by $\gamma_{2+} = (1 - \Gamma)Z$ and $\gamma_{2-} = -\Gamma Z$, respectively (Casassa and Eisenberg, 1960).

Titration studies (C. R. Hartzell, to be published) give a charge of $-11$ ($\pm 1$) for component 8 at pH 6.9 and of $-13$ ($\pm 1$) at pH 9.2. If it is assumed that $i = 1$, the values of $\gamma_{2+}$ and $\gamma_{2-}$ in 0.05 M phosphate at pH 6.9 are $-5.1$ and $+4.9$, respectively, while in 0.01 M borate at pH 9.2, $\gamma_{2+} = -7.4$ and $\gamma_{2-} = +5.6$. From the number of ions which have to be included in the macromolecular component defined according to Casassa and Eisenberg under the two conditions one can estimate the difference in the appropriate partial specific volumes by calculating the concentration of macromolecular component which produces the same density increment under the two sets of conditions. When the partial specific volume at pH 6.9 is 0.715 ml/g the above calculation gives a value of 0.711 ml/g at pH 9.2. This difference would give rise to a difference of just under 1.5% in measured molecular weights, which is rather better than the experimental precision of their measurement. It is concluded that any variation in the partial specific volume required by the Casassa and Eisenberg definition at pH 9.2 in 0.01 M sodium borate from that measured at pH 6.9 in 0.05 M sodium phosphate is not significant in comparison with experimental error.

The partial specific volumes of the other proteins used were taken as 0.75 ($\beta$-lactoglobulin and ovalbumin) and 0.728 ml/g (ribonuclease) (Tanford, 1963).

For the low-speed sedimentation equilibrium experiments where it is necessary to know the initial concentrations of the protein solutions, refractive index increment measurements were made as described in the preceding paper (Jeffrey, 1968).

A value of 0.00188 (g/100 ml)$^{-1}$ was used to convert concentrations in Rayleigh interference fringes, $j$, into concentrations in g/100 ml for the component 8 solutions. This was measured for reduced and alkylated low-sulfur protein fractions from wool in phosphate–sodium chloride buffer (pH 6.7), ionic strength 0.2, by Harrap and Woods (1958a). The protein concentrations used in their work were determined from dry weights measured by evaporating to dryness aliquots of protein solution and buffer which had been equilibrated by dialysis. In view of the small differences in the distribution of diffusible ions across the membrane at dialysis equilibrium referred to in the discussion of partial specific volumes, it is unlikely that the specific refractive increment at pH 9.2 in 0.01 M borate would differ measurably from that quoted above. A difference in the value taken for this parameter would not affect the qualitative interpretation of the results of the sedimentation equilibrium experiments but would merely change the absolute values of the equilibrium constants by shifting the measured molecular weights to slightly higher or lower weight concentrations. The refractive index differences actually measured in the experiments (in terms of Rayleigh interference fringes) were consistent with the Casassa and Eisenberg definition because the experiments were performed with solution and solvent equilibrated by dialysis.

*Sedimentation Equilibrium Experiments.* The experiments were performed in a Spinco Model E analytical ultracentrifuge and the Rayleigh interference optical system with a symmetrically placed upper limiting aperture was used. The experimental technique was exactly as described in the preceding paper.

Although the buffer salt concentration was only 0.01 M in these experiments, no evidence of convection either as spikes or anomalies in the concentration gradients was found in the MD experiments. Sodium borate has a reasonably high density (about 2 g/ml) and apparently provides enough density stabilization even at such low concentration.

Apparent weight-average molecular weights were calculated from eq 1 and 2, where $R$ is the gas constant,

$$\bar{M}_{\mathrm{wapp}(r)} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2}\left(\frac{\mathrm{d}\ln c}{\mathrm{d}(r^2)}\right)_r \quad (1)$$

$$\bar{M}_{\mathrm{wapp}\frac{(c_\mathrm{b} + c_\mathrm{a})}{2}} = \frac{c_\mathrm{b} - c_\mathrm{a}}{c_0(r_\mathrm{b}^2 - r_\mathrm{a}^2)}\frac{2RT}{(1 - \bar{v}\rho)\omega^2} \quad (2)$$

$T$ is the absolute temperature, $\bar{v}$ is the partial specific volume of the protein, $\rho$ is the density of the solvent, $\omega$ is the angular velocity, $c_\mathrm{b}$ and $c_\mathrm{a}$ are the concentrations at the cell bottom, $r_\mathrm{b}$, and meniscus, $r_\mathrm{a}$, respectively, and $c_0$ is the initial concentration.

For the LS experiments d ln $c$/d($r^2$) was evaluated by drawing tangents to large-scale plots of ln $j$ *vs.* $r^2$. This procedure was thought to be preferable to fitting the data with a least-squares regression and subsequently differentiating as there is some evidence that this may bias the molecular weight concentration relationship (Jeffrey and Coates, 1966). For the MD experiments d ln $c$/d($r^2$) was evaluated from the expression given by Yphantis (1964).

$$\left(\frac{\mathrm{d}\ln j}{\mathrm{d}(r^2)}\right)_{r_0} = \frac{0.1}{2r_0\Delta r}(2\ln j_{r_2} + \ln j_{r_1} - \ln j_{r_{-1}} - 2\ln j_{r_{-2}})$$

where $\ln j_{r_2} \ldots \ln j_{r_{-2}}$ are five data points spaced at equal increments $\Delta r$. No fringe displacements of less than 100 $\mu$ were used.

The apparent weight-average molecular weights measured in this work refer to the protein component as defined by Casassa and Eisenberg (1964) because the sedimentation equilibrium experiments were carried out with protein solution and solvent which had been equilibrated by dialysis and the partial specific volume was measured in a consistent manner. That such molecular weights are measured is subject of course to the conditions defined by Casassa and Eisenberg in their treat-

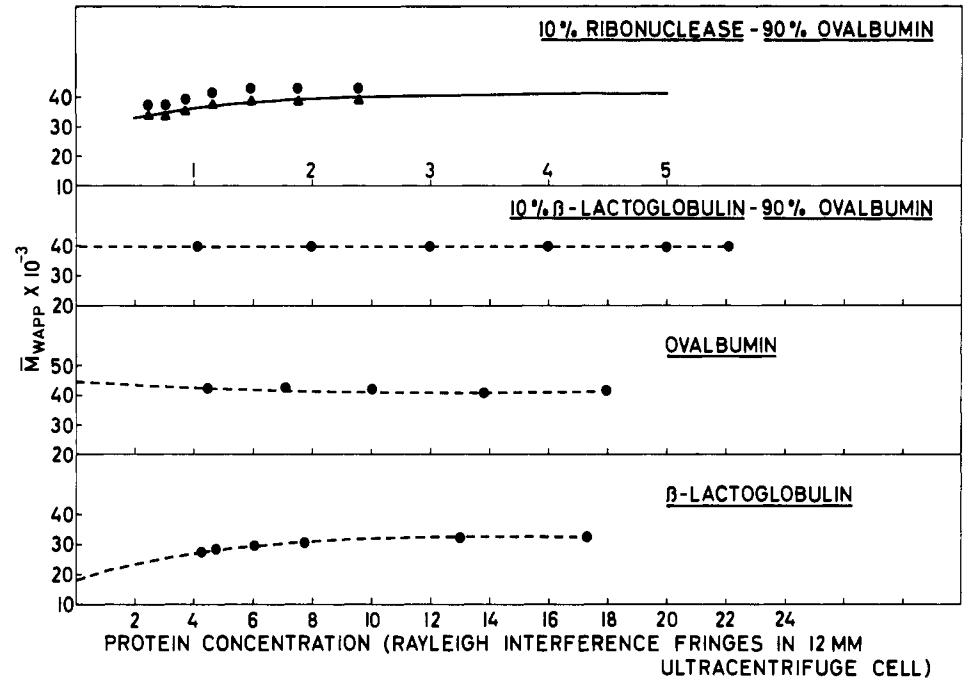

FIGURE 1: Sedimentation equilibrium experiments with mixtures of proteins in 0.01 M sodium borate (pH 9.2), 25°. The upper figure is an MD experiment at 39,460 rpm and initial protein concentration 0.15%. (●) Calculated using $\bar{v}$ for ovalbumin (0.75 ml/g). (▲) Calculated using $\bar{v}$ for ribonuclease (0.728 ml/g). The solid line is the calculated distribution for these experimental conditions. The lower three figures are for LS experiments at 16,200 rpm and initial protein concentrations 0.25%.

ment. The most important of these from the point of view of the present experiments are that compressibility effects are small and that long-range electrostatic forces can be neglected. The first of these conditions may be in doubt because of the comparatively high speeds (for sedimentation equilibrium experiments) at which the MD experiments were carried out. However the agreement between results from experiments at different speeds (see Results) implies that the neglect of compressibility effects is justified. Satisfaction of the second condition is perhaps questionable because of the low concentration (0.01 M) of salt. This concentration was selected because it is thought that the presence of salt favors association and would thus obscure the dissociation being investigated. The crucial point is whether at such a low salt concentration the apparent weight-average molecular weight curve extrapolated to infinite dilution gives the correct value for the monomer molecular weight. If the conditions for the Casassa and Eisenberg definition are satisfied the extrapolated molecular weight corresponds to that component of which the partial specific volume was measured. In the present instance the contribution to the molecular weight of the ions to be included in this component is only +272 g/mole (mol wt 23,000). If one considers the extrapolated value which would be obtained from measurements of the apparent weight-average molecular weight *vs.* concentration of a charged polymer in a polar solvent of very low or zero salt concentration, the treatment of Erlander (Erlander, 1963; Erlander and Senti, 1964) shows that the relationship, $M^{ext} = M_P - Z_P M_B$, holds where $M^{ext}$ is the extrapolated value, $M_P$ is the true molecular weight, $Z_P$ is the electrostatic charge, and $M_B$ is the molecular weight of a low molecular weight electrolyte. For a protein of charge −13 in a solution of sodium borate where the ions present are $Na^+$ and $B(OH)_4^-$ (Edwards *et al.*, 1955) $M^{ext} - M_P = -Z_P M_B = 13 \times 102 = 1326$. This is of the same order as the precision of the extrapolation (about ±2000) and so as well as can be determined experimentally the extrapolated value for the molecular weight of the monomer is that required by the Casassa and Eisenberg definition. The fact that the extrapolated molecular weight of ovalbumin in 0.01 M sodium borate at pH 9.2 is found to be 44,000 (see Results) lends support to this view. With a molecular weight of 44,000 and a charge between −12 and −20 at pH 9.2 (Longsworth, 1941), ovalbumin is comparable with component 8 of mol wt 23,000 and a charge of −13.

## Results

Apparent weight-average molecular weights rather than their reciprocals will be plotted in this paper because an associating system is being dealt with and a linear relationship between the reciprocal of the measured molecular weight and concentration is thus not expected. Figure 1 shows the results of LS experiments with ovalbumin, β-lactoglobulin, and a mixture of 10% β-lactoglobulin–90% ovalbumin in 0.01 M sodium borate. The experiment with ovalbumin shows slight nonideality and gives a molecular weight of 44,000 at infinite dilution, in good agreement with the value accepted for this protein (Gutfreund, 1944; Edsall, 1953).

The molecular weight *vs.* concentration data for β-lactoglobulin indicates that at pH 9.2, ionic strength 0.01, the protein dissociates into units of 18,000. This is in substantial agreement with the recently published results of McKenzie *et al.* (McKenzie and Sawyer, 1967; McKenzie *et al.*, 1967), although no sign of their time-dependent secondary transition was detected in this single experiment. The results for the mixture which was run to test the sensitivity of the sedimentation equilibrium technique to heterogeneity are interesting in relation to experiments in 8 M urea solutions described in the previous paper (Jeffrey, 1968). For the experiment in the absence of urea the extrapolated value of the molecular weight is 40,000 and the only sign of heterogeneity is in the ln $j$ *vs.* $r^2$ plot which is linear when it would be expected to be concave to the abscissa because of nonideality. These are the same indications obtained from an experiment with a mixture of the same composition in a solution containing 8 M urea (Jeffrey, 1968); however, in the present instance the sedimentation equilibrium experiment was at 16,200 rpm as compared with 39,460 rpm in 8 M urea and the $\beta$-lactoglobulin is mostly of mol wt 36,000 as compared with 18,000. This suggests that the sensitivity of detection of heterogeneity is much higher in the absence of urea. Figure 1 also shows the results of an experiment with 10% ribonuclease–90% ovalbumin at 39,460 rpm, the speed at which the MD experiments were carried out. Ribonuclease was used as a nonassociating protein having a molecular weight near that of suspected possible contaminants of component 8 (Jeffrey, 1968). At 39,460 rpm $\sigma$, defined by Yphantis (1964) as

$$\sigma = \frac{\omega^2 M(1 - \bar{v}\rho)}{RT} \qquad (3)$$

has a value of about 7.5 for ovalbumin in the absence of urea. This is above the value required for satisfaction of the condition that the protein concentration in the region near the meniscus be essentially zero at sedimentation equilibrium. $\sigma$(ovalbumin)/$\sigma$($\beta$-lactoglobulin) = 2.4 in 8 M urea and $\sigma$(ovalbumin)/$\sigma$(ribonuclease) = 3.1 in the absence of urea, and the experiments with 10% of the species of lower molecular weight are thus comparable. The results of the experiment with ribonuclease and ovalbumin show good agreement with the curve calculated for an ideal solution at this speed. The ln $j$ *vs.* $r^2$ plot has two linear regions, the lower one of which gives a molecular weight of 33,000 and the upper one 46,000. There is thus no difficulty in detecting the presence of 10% of species of the lower molecular weight in the absence of urea, and the sensitivity of the method seems to be close to that predicted by Yphantis (1964) for similar experimental conditions.

Figure 2 shows apparent weight-average molecular weights measured in MD and LS experiments with sample A of component 8. Both types of experiment were done in duplicate, and it can be seen from the figure that the MD experiments yield the type of molecular weight *vs.* concentration relationship characteristics of an associating system with a minimum molecular weight of about 20,000. However the results from the MD and LS experiments fail to superimpose satisfactorily and the most probable reason for this is heterogeneity (in the sense of a nonreacting mixture) of molecular weight which is emphasized at the higher speed. The presence of about 10% of species of molecular weight about 20,000 was suggested to account for the results obtained with sample A in 8 M urea (Jeffrey, 1968), and the results shown in Figure 2 are consistent with this explanation.

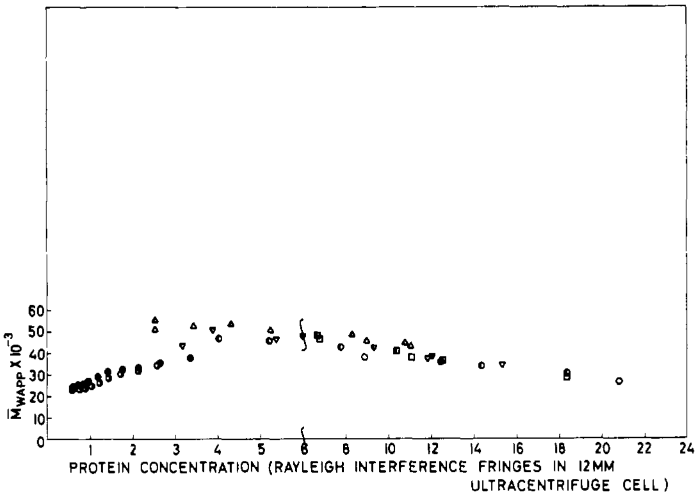

FIGURE 2: Sedimentation equilibrium experiments with sample A of component 8 in 0.01 M sodium borate (pH 9.2), 25°. (● and ◑) Duplicate MD experiments at 39,460 rpm and initial concentration 0.15%. (○▽△ and ◐▽◭) Duplicate LS experiments of different initial concentrations and 16,200 rpm. (□ and ◧) Molecular weights evaluated from eq 2.

In Figure 3 the apparent weight-average molecular weights obtained from MD and LS experiments with sample S of component 8 are given. There is better superposition of the results obtained from the experiments at different speeds, and moreover the results of MD experiments at different initial concentrations are in agreement. Together with the shape of the curve these results are good evidence for the existence of a reversible equilibrium, rapid with respect to the rate of separation of the species by the centrifugal field, between a monomer unit and one or more of its polymers. Sample S was thought to have a small amount (5% or less) of species of lower molecular weight present on the basis of experiments in 8 M urea solutions (Jeffrey, 1968). There does not seem to be enough of such an impurity to cause lack of superposition of the experiments at different speeds and initial concentrations in the absence of urea. There is, however, some indication of the presence of a small quantity of lower molecular weight material not participating in the equilibrium in that the MD experiment at 52,640 rpm yields molecular weights which tend to be slightly lower than those obtained at 39,460 rpm. The good agreement between experiments at different speeds provides evidence for the absence of the effect of pressure on the equilibrium, a possibility which should be taken into account in sedimentation experiments with associating systems (Kegeles *et al.*, 1967).

From Figure 3 the molecular weight of the monomer is 23,000 ± ~2000, that is, half of the molecular weight of the species found to be present in solutions containing 8 M urea. The pronounced negative slope of the higher concentration region of the $\bar{M}_{\mathrm{wapp}}$ *vs.* concen-

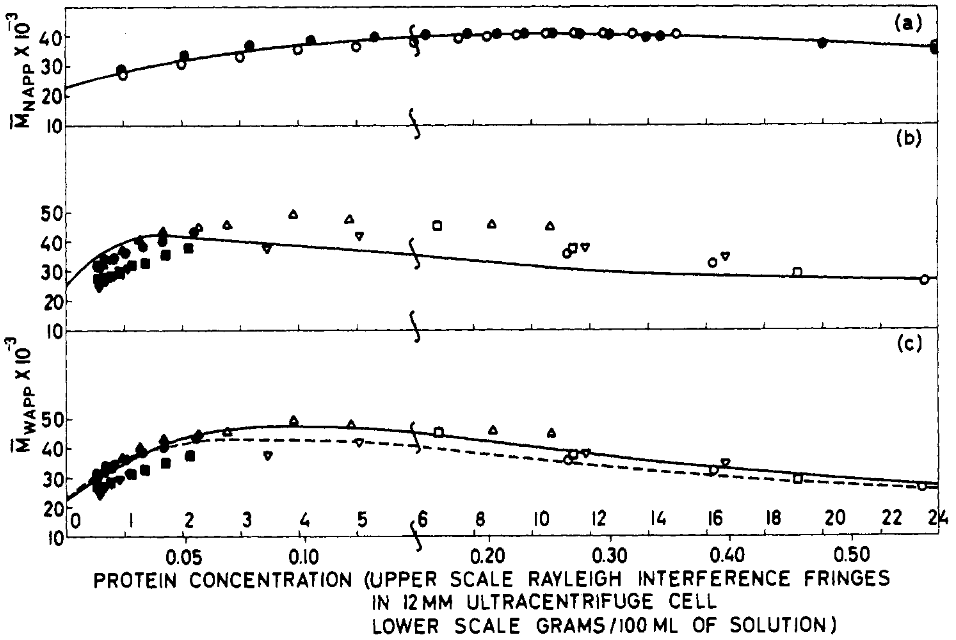

FIGURE 3: Sedimentation equilibrium experiments with sample S of component 8 in 0.01 M sodium borate (pH 9.2), 25°. (a) The solid line is the apparent number-average molecular weight evaluated experimentally (eq 12a). (●) Calculated for monomer–trimer (eq 12b). (○) Calculated for monomer–dimer–trimer (eq 12c). $M_1$ = 23,000, values of constants given in Table I. (b) (○▽△) LS experiments at different initial concentrations and 16,200 rpm. (□) Molecular weights evaluated from eq 2. (●) MD experiment at 39,460 rpm, initial concentration 0.09%. (▲) MD experiment at 39,460 rpm, initial concentration 0.03%. (■) MD experiment at 39,460 rpm, initial concentration 0.08%. (▼) MD experiment at 52,640 rpm, initial concentration 0.15%. The solid line is calculated for a monomer–dimer system with $M_1$ = 25,000, $K_2$ = 32.3, and $BM_1$ = 0.45 (eq 11a). (c) The symbols have the same significance as for b. The solid line is calculated for a monomer–trimer equilibrium (eq 11b), the dashed line for monomer–dimer–trimer (eq 11c) with $M_1$ = 23,000. The values of the constants are given in Table I.

tration plots shows that component 8 solutions in 0.01 M sodium borate are thermodynamically nonideal at such concentrations. Such nonideality has to be taken into account in any further analysis of the results.

Adams (1965) and Adams and Filmer (1966) have shown that it is possible to determine what polymeric species are present and the equilibrium constants between them in nonideal associating systems of macromolecules from the apparent weight-average molecular weight *vs.* concentration data. Their treatment is applicable when the macromolecules are charged if the species are defined in the Casassa and Eisenberg manner if it is assumed that the charges on the polymers ($M_N = iM_1$, where $M_1$ is the monomer molecular weight) are given by $iZ$, where $Z$ is the charge on the monomer. This assumption is reasonable and has to be made if one is to proceed any further. The molecular weights presented here have been measured in a way consistent with the Casassa and Eisenberg definition (see Methods), and in what follows the macromolecular component is understood to be defined in this way.

The first step in analyzing the results is to decide on the molecular weight of the monomer. The experimental results suggest that 23,000 is the best value; this seems reasonable as being half the value of 46,000 found for component 8 in 8 M urea solutions. However if the monomer molecular weight is taken as 23,000 the possibility of a monomer–dimer equilibrium is already eliminated because apparent molecular weights above 46,000 have been measured and the nonideality of the system tends to make the measured values low rather than high. Initially, then, the monomer molecular weight was taken as 25,000; this is within experimental error of 23,000 and allows the monomer–dimer hypothesis to be tested. The first quantity which can be obtained from the molecular weight *vs.* concentration data is the weight fraction of monomer as a fraction of concentration. Steiner (1952) showed that for an ideal system of associating macromolecules this is given by

$$\ln f = \int_0^c \left(\frac{M_1}{\bar{M}_{\text{w}-1}}\right)\frac{dc}{c} \tag{4}$$

where $f = c_1/c$ = the weight fraction of monomer at the concentration $c$, $M_1$ is the monomer molecular weight, and the nomenclature of Adams and Filmer (1966) is used. These authors have shown that in a non-ideal system

$$\ln f_a = \int_0^c \left(\frac{M_1}{M_{\text{wapp}}} - 1\right)\frac{dc}{c} = \ln f + BM_1c \tag{4a}$$

where $f_a$ might be called the apparent weight fraction of monomer and $B$ is the second virial coefficient. In the present work the integral in eq 4a was evaluated as a function of $c$ by trapezoidal integration. The values of $\bar{M}_{\text{wapp}}$ were taken at equal concentration intervals from a smooth curve drawn through the experimental points and about 30 points were used. The concentrations were converted from Rayleigh interference fringes into grams of macromolecular component/100 ml of solution by the use of the specific refractive index increment discussed under Methods. The value of $(M_1/\bar{M}_{\text{wapp}} - 1)/c$ is undefined at $c = 0$ so that in carrying out the trapezoidal integration one begins with the value of this quantity at the first value of the concentration chosen, $c_1$, thus leaving the value of the integral between $c = 0$ and $c = c_1$ unaccounted for. This was subsequently obtained by plotting the first differences of the values of the integral obtained *vs.* $c$ and extrapolating to $c = c_1$. The value obtained is then added to the integrals previously calculated. Having evaluated $f_a$ as a function of $c$, the quantity $\alpha$ (Adams and Filmer, 1966) can be calculated for each value of $c$.

$$\alpha = cf_a = c_1e^{BM_1c} \qquad (5)$$

Equilibria considered are listed in eq 6–9.

Monomer–dimer (Adams and Filmer, 1966)

$$2c = \frac{1}{\dfrac{M_1}{M_{\text{wapp}}} - BM_1} - \alpha e^{-BM_1c} \qquad (6)$$

Monomer–trimer (Adams, 1965)

$$3c = \frac{1}{\dfrac{M_1}{cM_{\text{wapp}}} - BM_1} + 2\alpha e^{-BM_1c} \qquad (7)$$

Monomer–dimer–trimer (Adams, 1965)

$$3c = \frac{1}{\dfrac{M_1}{cM_{\text{wapp}}} - BM_1} + 2\alpha e^{-BM_1c} + (L + BM_1)\alpha^2e^{-2BM_1c} \qquad (8)$$

where $L = K_2 - BM_1$ and is obtained from

$$\lim_{c\to 0}\left(\frac{M_1}{M_{\text{wapp}}} - 1\right)\Big/c = -K_2 + BM_1 \qquad (9)$$

The only unknown in eq 6–8 is $B$, and one considers each of the equilibria in turn trying different values of $B$ until a value is found which makes the right-hand side of the equation equal to the experimentally determined left-hand side.

In the present work the approach was for each postulated equilibrium to take two concentrations, one at each end of the concentration range, and to successively approximate $BM_1$ until a value was found which satisfied the appropriate equation for both values of the concentration. The value of $BM_1$ was then used to calculate the value of the equilibrium constant at each of the two concentrations. If the two values differed the mean was taken, and with the molecular weight of the monomer and $BM_1$ was used to generate a calculated $\bar{M}_{\text{wapp}}$ *vs.* concentration curve which was compared with the experimental points over the entire concentration range. The equilibrium constants are defined as

$$Kn = a_n/a_1^n = c_n/c_1^n \quad n = 2, 3 \qquad (10)$$

since $y_n/y_1^n = 1$ as a consequence of the assumption made by Adams (1965) in his treatment where the $\alpha$'s are activities and the $y$'s activity coefficients on the weight/volume concentration scale. The equations given below were used to calculate the $M_{\text{wapp}}$ curves from the equilibrium constants and $BM_1$.

Monomer–dimer

$$\frac{M_1}{M_{\text{wapp}}} = \frac{c}{c_1 + 2K_2c_1^2} + BM_1c \qquad (11a)$$

Monomer–trimer

$$\frac{M_1}{M_{\text{wapp}}} = \frac{c}{c_1 + 3K_3c_1^3} + BM_1c \qquad (11b)$$

Monomer–dimer–trimer

$$\frac{M_1}{M_{\text{wapp}}} = \frac{c}{c_1 + 2K_2c_1^2 + 3K_3c_1^3} + BM_1c \qquad (11c)$$

The calculated curve for monomer–dimer with a monomer molecular weight of 25,000 is shown in Figure 3b. It can be seen that it is not a very good fit to the points. Curves were also calculated for monomer–trimer and monomer–dimer–trimer and both cases were found to fit the experimental points quite well with monomer–trimer giving slightly the better fit over the whole concentration range. It should be pointed out that it is difficult to evaluate accurately the quantity $L$ (eq 8) which is required for analysis of the monomer–dimer–trimer case as it depends on extrapolation of $(M_1/M_{\text{wapp}} - 1)/c$ which is steep at low concentrations. The value of $L$ which was used in these calculations was obtained by taking the mean of the highest and lowest values it was possible to get from the extrapolation.

Since monomer–dimer is excluded on the basis of not giving a sufficiently good fit to the experimental data there is no need to use 25,000 for the molecular weight of the monomer when 23,000 appears a more reasonable value, and accordingly the whole calculation was repeated for the monomer–trimer and monomer–dimer–trimer using 23,000 as the monomer molecular weight. The constants found to give the best fit to the data for the conditions described are listed in Table I.

The curves calculated for the two equilibria from the values of the constants given in Table I for a monomer molecular weight of 23,000 are shown together with the experimental points in Figure 3c. The curves calculated for a monomer molecular weight of 25,000 are not shown but fit the data equally well. It is difficult to decide from the fit of the calculated curves to the experimental points whether monomer–trimer or monomer–dimer–trimer is the better description of the system although the monomer–trimer case does seem better in the middle of the concentration range. In an attempt to resolve the two cases apparent number-average mo-

TABLE I: Equilibrium Constants[a] and Virial Coefficients for Component 8 in 0.01 M Sodium Borate (pH 9.2).

| Item | Monomer Mol Wt 25,000 |  | Monomer Mol Wt 23,000 |  |
|---|---|---|---|---|
|  | Monomer–Trimer | Monomer–Dimer–Trimer | Monomer–Trimer | Monomer–Dimer–Trimer |
| $K_2$ |  | 13.3 |  | 19.9 |
| $K_3$ | 850 | 400 | 1700 | 1000 |
| $BM_1$ | 0.90 | 0.75 | 0.81 | 0.73 |

[a] Equilibrium constants are defined in eq 10 and are on the g/100 ml scale.

lecular weights, $\bar{M}_{n\,\mathrm{app}}$, were evaluated from the experimental data from the expression (Adams, 1965)

$$\frac{cM_1}{\bar{M}_{n\,\mathrm{app}}} = \int_0^c \frac{M_1}{\bar{M}_{w\,\mathrm{app}}}dc \qquad (12a)$$

and compared with number-average molecular weights calculated from

$$\frac{cM_1}{\bar{M}_{n\,\mathrm{app}}} = c_1 + \frac{K_3c_1^3}{3} + \frac{BM_1}{2}(c_1 + K_3c_1^3)^2 \qquad (12b)$$

for monomer–trimer and

$$\frac{cM_1}{\bar{M}_{n\,\mathrm{app}}} = c_1 + \frac{K_2c_1^2}{2} + \frac{K_3c_1^3}{3} + \frac{BM_1c^2}{2} \qquad (12c)$$

for monomer–dimer–trimer.

The results are shown in Figure 3a and obviously both equilibria describe the experimental results equally well. It is perhaps not surprising that it is hard to distinguish between the equilibria when the values of the constants shown in Table I are considered. For both the 23,000 and the 25,000 monomer, the equilibrium constant for trimer formation is about 30–50 times that for dimer formation in the monomer–dimer–trimer case. The weight fractions of the species present are shown for the two equilibria in Figures 4 and 5.

## Discussion

There is little to choose between the monomer–trimer and the monomer–dimer–trimer hypothesis on the basis of the ability of either to describe the experimental results. In the latter system the trimer is highly favored over the dimer (Table I and Figure 5), and it seems reasonable to conclude that the system is essentially a monomer–trimer equilibrium containing a very small proportion of dimer. The success of both schemes in fitting the results would reflect the small proportion of dimer present.

The schlieren pattern from a sedimentation velocity experiment with a rapidly reversible monomer–trimer system should show apparent resolution into two peaks (see, for example, Nichol *et al.*, 1964). In the present case examination for such apparent resolution is complicated by two factors. Firstly, the high value of the equilibrium constant for trimer formation means that

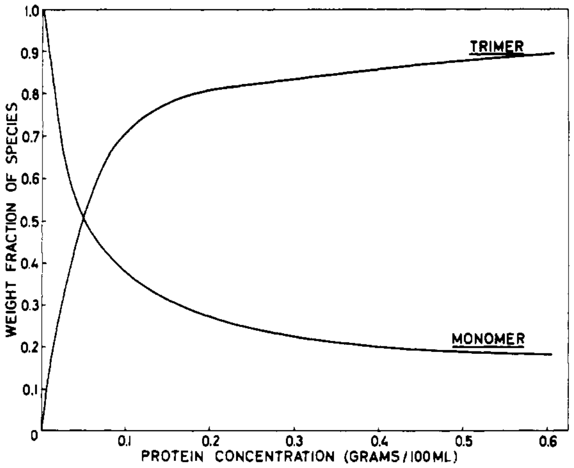

FIGURE 4: Weight fractions of monomer and trimer as a function of concentration for component 8 in 0.01 M sodium borate (pH 9.2), 25°. $M_1$ = 23,000; values of the constants given in Table I.

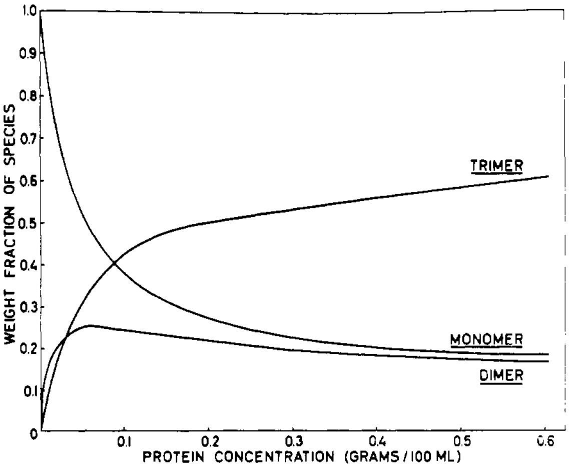

FIGURE 5: Weight fractions of monomer, dimer, and trimer as a function of concentration for component 8 in 0.01 M sodium borate (pH 9.2), 25°. $M_1$ = 23,000; values of the constants given in Table I.

the slow peak will be very small compared with the fast peak. Secondly, the high concentration dependence at finite concentrations (Figure 3c) will have the effect of sharpening the trailing side of the boundary, thus obscuring any resolution still further. The results of three sedimentation velocity experiments with component 8 solutions of concentrations 0.2, 0.4, and 1.4% in 0.01 M sodium borate are shown in Figure 6 together with the relevant experimental details. These experiments show the expected features, subject to the conditions mentioned above. That is, at the highest concentration the peak is sharpened on the trailing side as expected from the concentration dependence; at the intermediate concentration the peak is slightly asymmetrical, showing a tendency to broaden at the trailing edge; while at the lowest concentration this trend is continued and apparent resolution is hinted at. Unfortunately, this is the lowest concentration at which the schlieren optical system can be effectively used and all that can be said is that the transport experiments are consistent with the interpretation based on the sedimentation equilibrium results, namely, that the system can be described as a pure monomer–trimer equilibrium or as one containing a small percentage of dimer. The latter seems a better description on the grounds that it is unlikely that trimer is formed exclusively by three-body collisions and in any so-called monomer–trimer system there is likely to be some dimer present.

The results given in Table I show that a difference of 2000 in the value used for the monomer molecular weight makes a difference of about a factor of 2 in the equilibrium constant $K_3$ both in the monomer–trimer and the monomer–dimer–trimer case. The mean of the the $K_3$'s for monomer–dimer–trimer is 700 ± 300 and for monomer–trimer 1280 ± 420. That is to say that experimental error can account for the differences in the equilibrium constant for trimer formation derived for the two schemes. It would seem that values of $K_2$ of 10–20, $K_3$ of 500–1000, and $BM_1$ of 0.7–0.8 are reasonable if one considers that the proportion of dimer is probably somewhat lower than that obtained from the evaluation of the monomer–dimer–trimer case and if experimental error is taken into account.

Experiments with component 8 in solutions containing 8 M urea (Jeffrey, 1968) showed that the protein was homogenous with mol wt 46,000 under such conditions. There was no evidence for dissociation into units of mol wt 23,000 in 8 M urea, and the reason for this is not understood. After extraction and storage in 8 M urea, component 8 has a helix content of about 50% in aqueous solution after removal of urea by dialysis (Thompson and O'Donnell, 1965) which falls to near zero again in 8 M urea. It may be that the stability of what is apparently the dimer in 8 M urea is related to this change in tertiary structure. DeDeurwaerder and Harrap (1965) in a sedimentation equilibrium and osmotic pressure study of SCMKA in 60% formamide obtained a minimum molecular weight of 23,000–25,000 and evidence of an equilibrium between either monomer and trimer or monomer, dimer, and trimer. At that time no method of dealing with nonideal associating systems was available and the above authors were unable to find a value for the equilibrium constant for monomer–trimer which would apply to all of their results and concluded that the equilibrium was probably between monomer, dimer, and trimer.

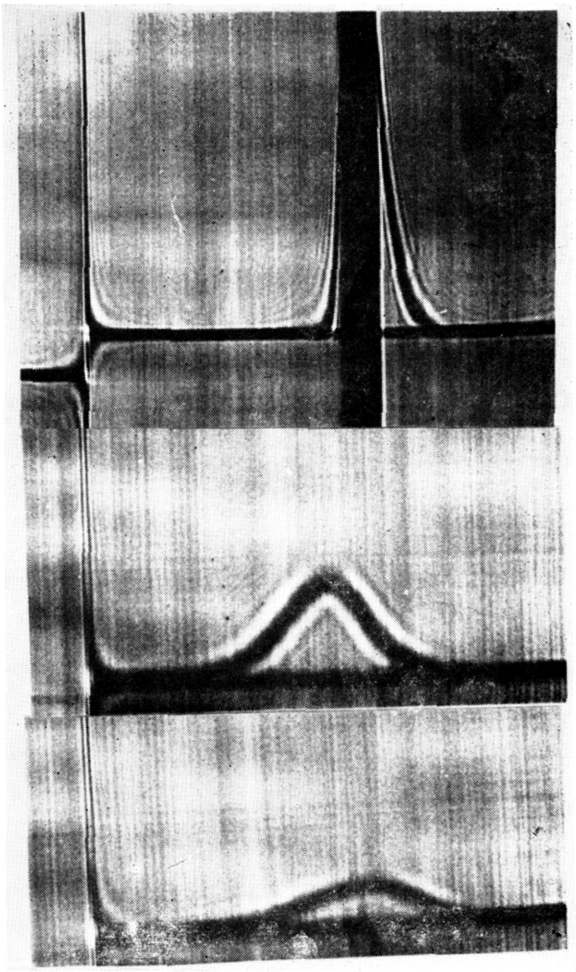

FIGURE 6: Sedimentation velocity experiments with component 8 in 0.01 M sodium borate (pH 9.20), 25°. Photographs at 70° bar angle. Upper: 1.4% speed 59,780 rpm, 10 min at set speed, synthetic boundary cell. Middle: 0.4% speed 67,770 rpm, 90 min at set speed. Lower: 0.2% speed 67,770 rpm, 90 min at set speed.

Failure of the monomer–trimer hypothesis to explain the results DeDeurwaerder and Harrap obtained with SCMKA probably reflects the mixed composition of the system (Thompson and O'Donnell, 1964, 1965) as also does the lack of superposition of sedimentation equilibrium experiments of different initial concentrations. Component 8 is a major constituent of SCMKA (Thompson and O'Donnell, 1964, 1965) and the results presented in the present paper, in particular the minimum molecular weight, are not inconsistent with those for SCMKA. Experiments with component 7, the other major constituent of SCMKA (Thompson and O'Donnell, 1964, 1965), are planned, and it is hoped that the results of these together with those for component 8 will lead to an explanation of the relationship between the proteins comprising the low-sulfur proteins of the wool fiber and their behavior both singly and together in solutions of different compositions.

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## References

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

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

Casassa, E. F., and Eisenberg, H. (1960), *J. Polymer Sci. 47*, 29.

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

DeDeurwaerder, R., and Harrap, B. S. (1964), *Makromol. Chem. 72*, 1.

DeDeurwaerder, R., and Harrap, B. S. (1965), *Makromol. Chem. 86*, 98.

Edsall, J. T. (1953), *Proteins 1*, 634.

Edwards, J. O., Morrison, G. C., Ross, V. F., and Schultz, J. W. (1955), *J. Am. Chem. Soc. 77*, 266.

Erlander, S. R. (1963), *Makromol. Chem. 65*, 91.

Erlander, S. R., and Senti, S. R. (1964), *Makromol. Chem. 73*, 14.

Gutfruend, H. (1944), *Nature 153*, 406.

Harrap, B. S., and Woods, E. F. (1958a), *Australian J. Chem. 11*, 581.

Harrap, B. S., and Woods, E. F. (1958b), *Australian J. Chem. 11*, 592.

Jeffrey, P. D. (1968), *Biochemistry 7*, 3345 (this issue; preceding paper).

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

Kegeles, G., Rhodes, L., and Bethune, J. L. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 45.

Longsworth, L. G. (1941), *Ann. N. Y. Acad. Sci. 41*, 267.

McKenzie, H. A., and Sawyer, W. H. (1967), *Nature 214*, 1101.

McKenzie, H. A., Sawyer, W. H., and Smith, M. B. (1967), *Biochim. Biophys. Acta 147*, 73.

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

O'Donnell, I. J., and Woods, E. F. (1956), *J. Polymer Sci. 21*, 397.

Steiner, R. F. (1952), *Arch. Biochem. Biophys. 39*, 333.

Tanford, C. (1963), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 358.

Thompson, E. O. P., and O'Donnell, I. J. (1964), *Australian J. Biol. Sci. 17*, 973.

Thompson, E. O. P., and O'Donnell, I. J. (1965), *Australian J. Biol. Sci. 18*, 1207.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.