Studies on Self-Association of Proteins. The Self-Association of α-Chymotrypsin at pH 8.3 and Ionic Strength 0.05†

M. W. Pandit* and M. S. Narasinga Rao‡

ABSTRACT: The self-association of α-chymotrypsin at pH 8.3 and μ = 0.05 was studied by measuring the weight-average molecular weight by the Archibald method and the sedimentation coefficient as a function of protein concentration. Considerable autolysis of the protein occurred under the experimental conditions used. A method was devised to measure the extent of autolysis. The molecular weight data were corrected for the presence of the autolysis product(s) which itself did not undergo self-association. An analysis of the experimental molecular weight data for various models of self-association showed that indefinite self-association model with nonideality term set to zero fitted the data the best. However, analysis of the sedimentation coefficient data showed that a monomer–hexamer equilibrium, without any intermediate species, fitted the data the best. Pressure in sedimentation velocity experiments did not affect $s_{20,w}$ values but affected the shape of the velocity patterns. The addition of autolysis product(s) of the enzyme did not affect self-association as revealed by molecular weight data, but affected the sedimentation velocity patterns.

The self-association of α-chymotrypsin (EC 3.4.4.5) under different conditions of pH and ionic strength has been studied (Schwert, 1949; Schwert and Kaufman, 1951; Smith *et al.*, 1951; Smith and Brown, 1952; Massey *et al.*, 1955; Tinoco, 1957; Egan *et al.*, 1957; Rao and Kegeles, 1958; Rao, 1961; Nichol and Bethune, 1963; Winzor and Scheraga, 1963, 1964; Ackers and Thompson, 1965; Sarfare *et al.*, 1966; Morimoto and Kegeles, 1967).

At an ionic strength of 0.1 and above essentially dimerization occurs. Rao and Kegeles (1958) showed that at pH 6.2 and μ[1] = 0.20 (phosphate buffer) dimers and trimers were formed. Winzor and Scheraga (1964) found that at pH 3.86 and μ = 0.20 (acetate buffer) dimers were formed. Massey *et al.* (1955) concluded from sedimentation velocity measurements in a phosphate buffer of pH 7.9 and $\mu \sim 0.03$ that extensive association occurred. Using the data of Massey *et al.* (1955), Gilbert (1955) showed that under these conditions a monomer–hexamer equilibrium, without the presence of any intermediate species, existed. Ackers and Thompson (1965) came to a similar conclusion from gel filtration studies.

Although it has been concluded that at low ionic strength extensive association occurs, the molecular weight as a function of protein concentration has not been measured to determine the degree of association, equilibrium constants of the reaction, the type of association etc. In this investigation the molecular weight of α-chymotrypsin was measured as a function of protein concentration in Tris buffer[1] of pH 8.3 and μ = 0.05, by the Archibald method. Parallel sedimentation velocity measurements were also made. The object of this investigation was (1) to analyze the data in terms of various models for the association reaction; and (2) to compare the results of Achibald and sedimentation velocity measurements.

Materials and Methods

α-Chymotrypsin. Worthington α-chymotrypsin, 3× crystalline, CDI 7-JC, was used without purification.

Chemicals. The chemicals used were guaranteed reagent grade or chemically pure grade.

CM-Sephadex Chromatography. CM Sephadex C-50 (from Pharmacia, Sweden) was regenerated by standard methods; it was then equilibrated with acetate buffer of pH 5.5 and 0.05 M and packed into a double-walled glass column, which was maintained at 5° by circulation of refrigerated water. The packed column dimensions were 10 × 1.4 cm. For elution of the protein a linear gradient of 0.05–1.00 M acetate was used. Fractions of 2 ml were collected on a GME fraction collector. The absorbance of the fractions at 280 nm was read on a Hilger Uvispek spectrophotometer using cells of 0.5-cm optical path.

Preparation of the Autolysis Product of α-Chymotrypsin. α-Chymotrypsin (500 mg) was dissolved in 10 ml of distilled water and the pH of the solution was adjusted to 7.8 with dilute ammonia solution. The mixture was then incubated with shaking at 37° in an air incubator. The pH of the solution was found to decrease with time in the initial stages of the reaction and was readjusted to pH 7.8 with the addition of ammonia solution every 30 min. After 24 hr the pH was found to be nearly constant. Incubation was continued for 72 hr. The mixture was then centrifuged at 3000 rpm for 15 min and the supernatant loaded on a CM-Sephadex column equilibrated with water. Elution was performed with distilled water and was followed by a LKB-Uvicord absorptiometer. When the absorption reading reached the base line after the peak, elution was stopped. The collected eluent solution was lyophilized and stored in the cold.

Archibald Molecular Weight. The molecular weight measurements were made with a Spinco Model E ultracentrifuge equipped with schlieren phase plate and absorption optics, and RTIC unit. The method of Rao and Kegeles (1958) was followed. For the false bottom, 0.1 ml of fluorocarbon oil (FC 43) was used. The rotor temperature was adjusted to 25° and was maintained during the run with RTIC unit. For experiments below protein concentrations of 5 g/l., a

† From the Biochemistry Division, Regional Research Laboratory, Hyderabad 500009, India. *Received June 20, 1973.* This is the first of a series of papers on the studies on the self-association of proteins.

‡ Present address: Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570013, India.

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; μ, ionic strength.

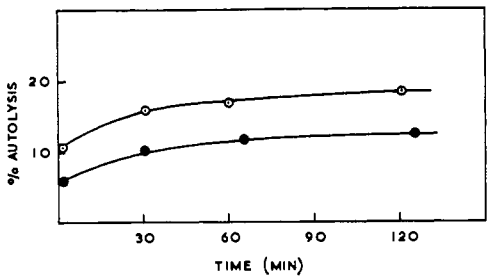

FIGURE 1: Rate of autolysis of α-chymotrypsin at 25° in Tris buffer of pH 8.3 and $\mu = 0.05$: (⊙) α-chymotrypsin alone; (●) α-chymotrypsin in presence of 20% autolysis product(s).

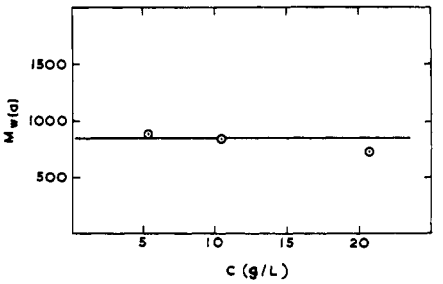

FIGURE 2: Molecular weight of the autolysis product as a function of concentration.

30-mm cell centerpiece was used. For other concentrations a standard 12-mm centerpiece was used. The speeds used ranged between 5784 and 15,220 rpm.

Sedimentation velocity measurements were also made at 25°.

Protein Concentration. This was determined spectrophotometrically using a value of 20.6 for $E^{1\%}_{280\ nm}$ (Rao and Kegeles, 1958).

Buffer Solution. Tris-HCl buffer of pH 8.3 and 0.02 M was prepared and the ionic strength made to 0.05 by the addition of KCl.

Results and Discussion

The homogeneity of α-chymotrypsin was checked by chromatography on CM-Sephadex. It gave a small peak (2–3% of the total) immediately after the void volume and another, fairly symmetrical, at 0.4 M acetate. The minor peak did not have enzyme activity; the activity was associated with the major peak. The minor peak was in all probability due to autolysis product(s) of the enzyme.

Molecular weight measurements were made at pH 8.3 since at this pH the protein would be isoelectric (Anderson and Alberty, 1948; Rao and Kegeles, 1958) and the effects of nonideality would be minimum. However, this pH is close to the pH optimum of the enzyme (Northrop *et al.*, 1948). It was observed that considerable autolysis of the enzyme occurred during the time interval needed for the molecular weight measurements; the measured molecular weights, at low concentrations of the enzyme, were lower than the minimum molecular weight of α-chymotrypsin reported. The addition of a competitive inhibitor of chymotrypsin, β-phenylpropionate, or $CaCl_2$ did not suppress the autolysis. It was therefore necessary to estimate the proportion of autolysis product(s) produced during experimentation and apply a correction to the molecular weight data.

In CM-Sephadex chromatography experiments at pH 5.5 and 0.05 M, the low molecular weight (autolyzed) material eluted immediately after the void volume and the macromolecular enzyme eluted at 0.4 M acetate. This chromatographic procedure was used to determine the rate of autolysis; 20 mg of protein was dissolved in 2 ml of Tris buffer and left at 25° for the desired interval of time. The reaction was "stopped" by loading 1 ml of the solution on CM-Sephadex column. It was then eluted with acetate buffer of pH 5.5 and 0.05 M, and 3-ml fractions were collected. Elution was continued till the absorbance of the solution at 280 nm was zero. The column was then eluted with 0.4 M acetate buffer and fractions were collected till the absorbance was zero. From the measurement of area under the two peaks in the chromatogram the proportion of the first peak was calculated as a function of incubation time. The recovery of the material from the column was better than 95%.

In Figure 1 the per cent of autolysis as a function of time is given. It was observed that after an interval of 30 min the proportion of the autolysis product(s) reached a steady value of 17%. The rate of autolysis was found to be independent of initial enzyme concentration up to 4%, a range of concentration which was used for molecular weight determination. The rate of autolysis, as was to be expected, was lowered by the prior addition of the autolysis product(s) to the incubation mixture. In the presence of 20% autolysis product(s), the *freshly produced* autolysis product was only 12% at the end of 120 min incubation (Figure 1).

For molecular weight determination the following procedure was followed. The protein solution in the buffer was left at 25° for 30 min and then loaded into the centrifuge cell. Photographs of the pattern were taken at 15, 30, and 45 min intervals after the attainment of the operating speed. From each photograph the molecular weight corresponding to the top and bottom meniscus was obtained. The values obtained at different intervals of time were extrapolated to zero time, using for extrapolation the value at the top and bottom meniscus separately. An average of the extrapolated values was taken. This procedure is the same as the one used by Sarfare *et al.* (1966) in their study of the association of α-chymotrypsin at pH 6.2 in phosphate buffer of ionic strength 0.2, in the presence of the added competitive inhibitor, β-phenylpropionate. To give an estimate of the time dependence of molecular weight and also the difference in the value at the top and bottom meniscus, raw data at three protein concentrations are given in Table I.

It was observed that the molecular weight of the autolysis product did not vary with its concentrations (Figure 2) suggesting that it did not undergo self-association reaction.

TABLE I: Molecular Weight at the Top and Bottom Meniscus as a Function of Time of Centrifugation.

Protein Concn (g/l.)	Time (min)	M_w Top Meniscus	M_w Bottom Meniscus
6.3	15	33,930	39,970
	30	32,120	41,980
	45	31,710	43,980
15.0	15	68,900	65,120
	30	66,400	67,810
	45	65,970	68,870
24.4	15	90,570	88,940
	30	89,950	89,780
	45	89,910	90,010

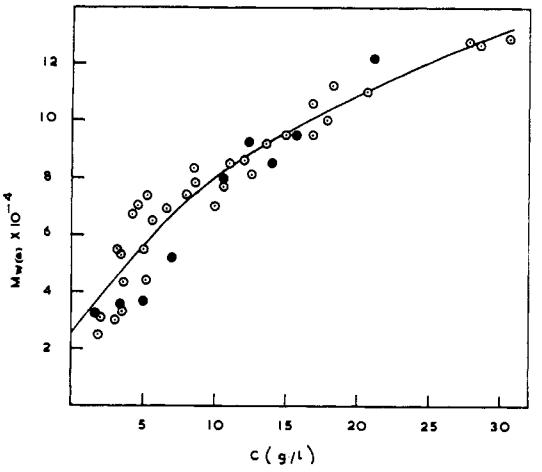

FIGURE 3: Apparent weight-average molecular weight of α-chymotrypsin as a function of protein concentration in Tris buffer of pH 8.3 and $\mu = 0.05$; (○) α-chymotrypsin alone; (●) α-chymotrypsin in presence of 20% autolysis product(s); (——) the best curve drawn through the experimental points.

The average of the values, obtained at three concentrations, was 850; for this calculation the partial specific volume of the autolysis product was assumed to be 0.75.

Since the autolysis product did not associate it was possible to apply a correction for the weight-average molecular weight obtained at different protein concentrations. The correction was applied in the following way

$$M_{w,obsd} = \frac{M_{w,true}C_P + M_AC_A}{C_P + C_A}$$

where $M_{w,obsd}$ is the observed weight-average molecular weight; $M_{w,true}$, the true weight-average molecular weight of the protein; C_p, concentration of the protein; M_A, molecular weight of the autolysis product; C_A, concentration of the autolysis product. Since C_T, the total concentration, is equal to $(C_P + C_A)$ it follows

$$M_{w,true} = \frac{M_{w,obsd}C_T - M_AC_A}{C_T - C_A}$$

It was observed in all the cases that the quantity, M_AC_A, was negligible in comparison with $M_{w,obsd}C_T$.

In Figure 3, the apparent weight-average molecular weight, $M_{w(a)}$, is given as a function of protein concentration, C, expressed in g/l. The protein concentration was also corrected for the presence of 17% autolysis product(s). A set of molecular weight measurements in the presence of 20% (on protein basis) added autolysis product(s) was also made. As mentioned earlier under these conditions 12% autolysis product(s) was produced. So the molecular weight values were corrected for 32% (20% + 12%) autolysis. The $M_{w(a)}$ values determined in the presence of 20% autolysis product(s), after correction, fit the same curve as the set of data obtained in the absence of added autolysis product(s). The agreement in the values gave us confidence that this method of applying correction was a reasonable one; further it indicated that the autolysis product(s) did not affect the self-association of α-chymotrypsin. Experiments in progress with DFP-chymotrypsin gave $M_{w(a)}$ *vs.* C data which fit the same curve.

$M_{w(a)}$ *vs.* C data were analyzed to determine the model of self-association which fit the data the best.

All the types of analysis entailed a knowledge of the molecular weight of α-chymotrypsin monomer. The best curve drawn through the experimental points and extrapolated to zero concentration gave $M_{w(a)} = 23{,}000$–$25{,}000$. This was in good agreement with the values determined by physical methods (Rao and Kegeles, 1958). The chemical molecular weight of α-chymotrypsin calculated from amino acid composition is 25,000 (Hartley and Kaufman, 1966). Rao and Kegeles (1958) used a value of 23,000 in their study of self-association of α-chymotrypsin at pH 6.2 and $\mu = 0.20$. Adams and Filmer (1966) also used the same value. To facilitate comparison with earlier calculations a value of 23,000 was used in these calculations also. It may be mentioned that the use of a value of 23,000 instead of 25,000 does not materially affect the conclusions drawn on the model of self-association.

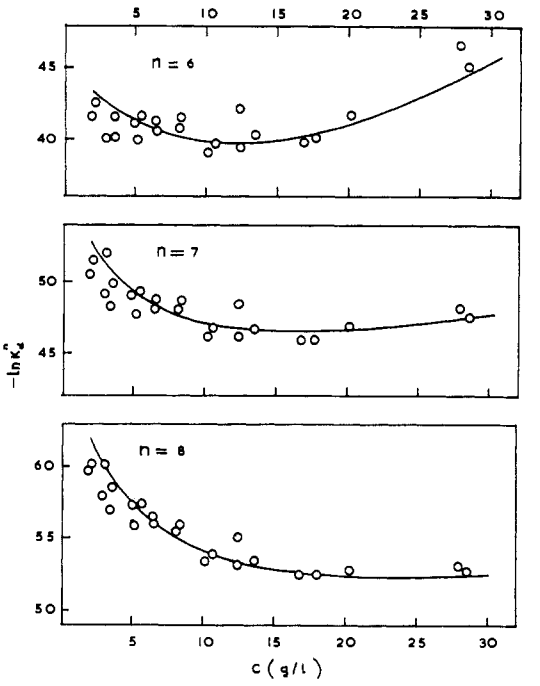

FIGURE 4: Plots for $-\ln K_d^n$ *vs.* C (g/l.) for $n = 6$, 7, and 8.

Monomer–n-mer Equilibrium Model. (a) Method of McKenzie et al. (1967). The equation used was

$$\log K_d^n = \log n + (n-1)\log(C/M_1) - (n-1) \times \log(nM_1 - M_1) + n\log(nM_1 - M_w) - \log(M_w - M_1) \quad (1)$$

where K_d^n is the *dissociation constant* on a mol/l. scale; n, the value of the n-mer; C, concentration in g/l.; M_1, the molecular weight of the monomer; and M_w, the measured molecular weight at C. When the right-hand side of eq 1 is plotted against C, it should yield a straight line parallel to the X axis, if n has been correctly chosen. The intercept would be $\log K_d^n$. In Figure 4, such plots for $n = 6$, 7, and 8 are given. The data did not give a straight-line plot for any value of n. However, for $n = 6$, if the experimental points at the two highest concentrations were omitted, the data could be reasonably fitted to a straight line, which was parallel to the X axis, in view of the scatter in the experimental points. Omitting these two values, an average value of $\log K_d^n$ ($= -17.65$) was obtained. Expressed as dissociation constant on concentration scale of g/l., this value would be $\log K_d^n = +3.37$. With this value $M_{w(a)}$ was calculated as a function of C from eq 1. The calculated curve, shown in Figure 5, did not fit the experimental data, especially in the region of high concentration.

(b) *Method of Adams (1967).* For a nonideal, monomer–*n*-mer equilibrium Adams (1965) has shown that

$$n = \left[\frac{1}{[M_1/CM_{w(a)}] - BM_1} - C_1\right]\Big/(C - C_1) \qquad (2)$$

where M_1 is the monomer molecular weight; $M_{w(a)}$, the apparent weight-average molecular weight at the concentration, C; BM_1, the nonideal term; C_1, the monomer concentration at C, corrected for nonideality.

Adams and Williams (1964) have also shown that

$$C_1 = \alpha \exp(-BM_1C) \qquad (3)$$

where α is monomer concentration not corrected for nonideality. α can be obtained by Steiner's method (Steiner, 1954) from $M_{w(a)}$ *vs.* C data. Substituting different values of BM_1, in eq 3 and hence values of C_1 in eq 2, n may be calculated as a function of C. For a nonideal monomer–*n*-mer equilibrium, n would be constant at all values of C for the correctly chosen value of BM_1.

α as a function of C was determined by Steiner's method (Steiner, 1952). In spite of the scatter in the experimental $M_{w(a)}$ *vs.* C data a reasonably smooth curve was obtained and α could be determined with a fair degree of confidence. With these values, the right-hand side of eq 2 was calculated as a function of C for different values of BM_1. None of the plots could be considered as linear and horizontal to the X axis (Figure 6). If the plot for $BM_1 = 0.005$ could be approximated to a straight line, a value of $n = 2$–3 would be obtained. This would be clearly incompatible with the experimental $M_{w(a)}$ data since the highest value ($\sim 12.0 \times 10^4$) was five times the monomer molecular weight.

(c) *Method of Gilbert.* The monomer–*n*-mer hypothesis can be tested from a different approach. In sedimentation velocity experiments bimodal peaks were obtained above a protein concentration of 5 g/l. The proportion of the fast peak increased with protein concentration. These observations were in agreement with those of Massey *et al.* (1955) and Nichol and Bethune (1963). The application of Gilbert's theory (Gilbert, 1955, 1959) would suggest that a monomer–*n*-mer model would be valid for this system. An apparent dissociation constant can be calculated from the areas under the two peaks in sedimentation velocity patterns. The dissociation constant, K, is given by the relation (Gilbert, 1955)

$$K = \frac{n(1 - \delta_{min})}{\delta_{min}} \frac{{C_{min}}^{n-1}}{\left[1 + \frac{1}{n}\frac{\delta_{min}}{1 - \delta_{min}}\right]^{n-1}} \qquad (4)$$

$$\delta_{min} = \frac{n - 2}{3(n - 1)} \qquad (5)$$

C_{min} corresponds to the concentration of the slower peak. Once K is known, M_w as a function of C can be calculated with the equation (Rao and Kegeles, 1958)

$$\frac{C^{n-1}}{K} = \frac{(n - 1)^{n-1}[(M_w/M_1) - 1]}{[n - (M_w/M_1)]^n} \qquad (6)$$

The sedimentation velocity patterns were enlarged and the areas were determined planimetrically. In Table II, the concentration of the slower peak, calculated from the areas, is given. Column 1 gives the total protein concentration. Since autolysis product(s) which formed 17% of the total protein were not aggregable the values in column 1 were multiplied by 0.83 to give the concentration of the aggregable material and are given in column 2. The fraction of the fast peak with respect to the total is given in column 3. Column 4 gives the product of the value in columns 1 and 3. This value subtracted from the value in column 2 gave the concentration of the slower peak and is shown in column 5. It was seen that this value was a constant, 4.04 ± 0.23 g/l. The fact that the pattern at protein concentration less than ~5 g/l. was unimodal gave support to this conclusion. Massey *et al.* (1955) reported a value of 1.75 g/l. and Nichol and Bethune (1963) a value of 3.5–3.9 g/l. The value obtained by us is nearly the same as that obtained by Nichol and Bethune (1963) who made measurements at pH 8.45 and $\mu = 0.05$ (barbiturate buffer).

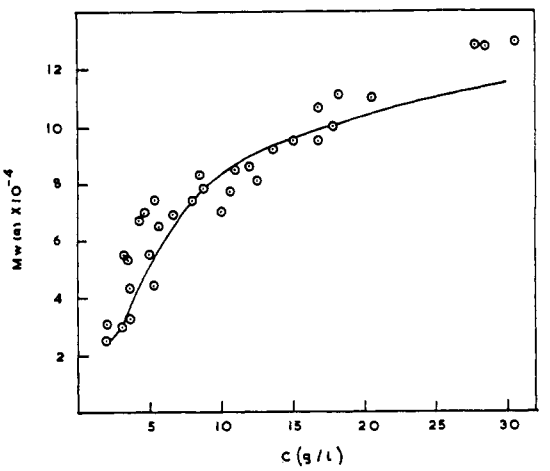

FIGURE 5: $M_{w(a)}$ *vs.* C curve calculated with $n = 6$ and $\log K_d{}^n = -17.65$; (⊙) experimental data.

With this value for the concentration of the slow peak, K was calculated for various values of n. The dissociation constant on g/l. concentration scale was $\log K_d = 4.12$ for $n = 6$ and $\log K_d = 4.77$ for $n = 7$. The calculated $M_{w(a)}$ *vs.* C curves for $n = 6$ or 7 are given in Figure 7. The curves did not fit the experimental data. Although the curve for $n = 7$ fitted the data above $C \sim 10$ g/l., it failed completely to fit the data below this concentration.

Nonideal Indefinite Self-Association Model. (a) Method of Adams (1967). The data were analzed for the indefinite self-association model using eq 7–11

$$\frac{CM_1}{M_{n(a)}} = \int_0^C \frac{M_1}{M_{w(a)}} dc \qquad (7)$$

$$C = C_1/(1 - KC_1)^2 \qquad (8)$$

$$\frac{M_1}{M_{w(a)}} = \frac{(1 - KC_1)}{(1 + KC_1)} + BM_1C \qquad (9)$$

$$\frac{M_1}{M_{n(a)}} = (1 - KC_1) + \frac{BM_1C}{2} \qquad (10)$$

$$\frac{M_1}{M_{w(a)}} = \frac{1}{\frac{2}{\frac{M_1}{M_{n(a)}} - \frac{BM_1C}{2}} - 1} + BM_1C \qquad (11)$$

where M_1 is the monomer molecular weight; $M_{w(a)}$, the apparent weight-average molecular weight; $M_{n(a)}$, the apparent number-average molecular weight; C, the protein concentration; C_1, the concentration of the monomer corrected for nonideality; K, the *association constant;* and BM_1, the nonideality term.

$M_1/M_{n(a)}$ as a function of C can be obtained by graphical integration of $M_1/M_{w(a)}$ *vs.* C data (Adams, 1967). Then eq 11

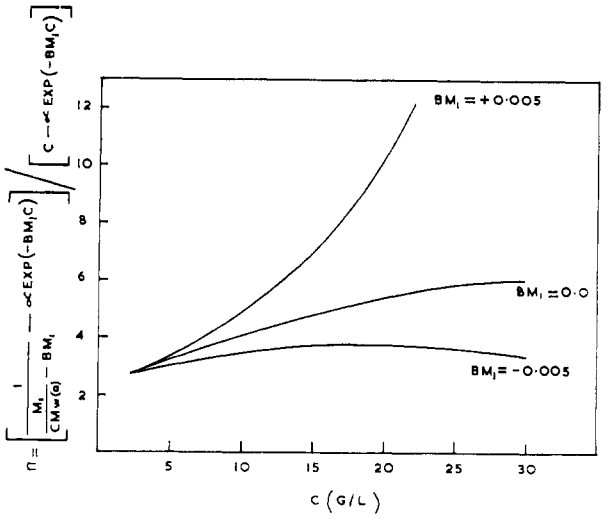

FIGURE 6: Calculated curves for n (Adams, 1967).

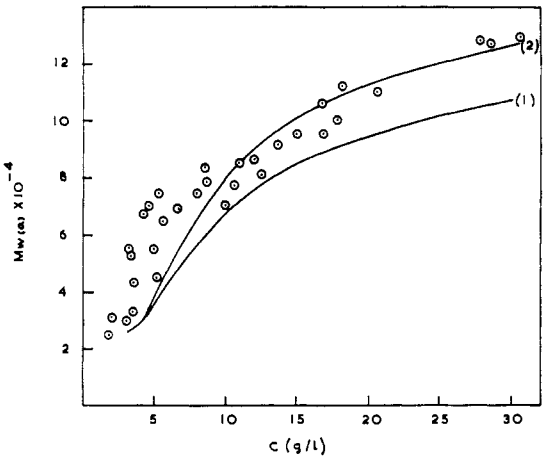

FIGURE 7: $M_{w(a)}$ *vs.* C curves calculated with dissociation constant obtained from sedimentation velocity patterns (1) $n = 6$; (2) $n = 7$; (⊙) experimental data.

contains only one unknown, BM_1, which can be solved by successive approximation. Once BM_1 is known, KC_1 can be obtained from eq 10 and $M_1/M_{w(a)}$ from eq 9. This can then be converted into $M_{w(a)}$ *vs.* C curve.

Such calculated curves with positive, negative, and zero value of BM_1 are shown in Figure 8. The curve for $BM_1 = 0$ fitted the data the best. The other curves ($BM_1 = +0.005$ or -0.005) failed completely to fit the data. The fit between the calculated curve (for $BM_1 = 0$) and the experimental data was satisfactory over the entire concentration range, unlike in the case of the curve calculated from monomer–n-mer model (Figure 7).

(*b*) *Method of Van Holde and Rossetti (1967).* Analysis for nonideal indefinite self-association model was also made by the method of Van Holde and Rossetti (1967). Their equation is

$$4K = \left[\frac{R_a^2}{(1 - BM_1CR_a)^2} - 1\right]\Big/C \qquad (12)$$

where $R_a = M_{w(a)}/M_1$. The calculations by this method did not need a derived quantity such as $M_1/M_{n(a)}$ and the experimental $M_{w(a)}$ *vs.* C data could be straight-away used in eq 12.

For the correctly chosen value of BM_1, the right-hand side of eq 12 when plotted against C would yield a straight line with zero slope and the intercept would be equal to $4K$. A straight line with zero slope was obtained for $BM_1 = 0$. From the intercept a value of $4K = 1.0$ could be obtained; this value expressed as a dissociation constant on a concentration scale of g/l. would be log $K_d = 0.6$. With $4K = 1.0$, M_w was calculated with eq 12. The calculated curve, shown in Figure 8, agreed with that calculated by the method of Adams (for $BM_1 = 0$) and also with the experimental data.

Discrete Self-Association Model. When association leads to the formation of a finite aggregate with all the intermediate species present in the equilibrium mixture, it is referred to as discrete self-association. The true weight-average molecular weight, $M_{w(c)}$, would be given by the relation

$$\frac{M_1}{M_{w(c)}} = \frac{C}{(C_1 + 2KC_1^2 + 3KC_1^3 + \cdots + nK^{n-1}C_1^n)} \qquad (13)$$

where $M_{w(c)}$ is the true weight-average molecular weight (corrected for nonideal term) and $C_1 = \alpha \exp(-BM_1C)$. The relation between $M_{w(a)}$ and $M_{w(c)}$ is given by the equation (Adams and Fujita, 1963)

$$\frac{M_1}{M_{w(a)}} = \frac{M_1}{M_{w(c)}} + BM_1C \qquad (14)$$

In writing eq 13 the assumption was made that all the association constants had the same value. This assumption was made for simplification of computer calculations. A computer program (in Fortran II language) was written for calculating $M_1/M_{w(a)}$ as a function of C, for which α and C from the experimental data were used; α was obtained by Steiner's method (Steiner, 1952). For any one set of calculations the values of n, K, and BM_1 were held constant. The values of n were varied from 5 to 10 using all the integers in between. K was varied from $+0.1$ to $+1.0$ with $\Delta K = +0.1$ and BM_1 from -0.10 to $+0.10$ with $\Delta BM_1 = +0.02$. The choice of the range of K and BM_1 values was guided by the values obtained by analyzing the data for indefinite self-association hypothesis described earlier. All possible sets of combination of K, BM_1, and n were tried. The computer calculations were made on a IBM 1620 computer.

The calculations showed that no set of n, K, and BM_1 would give a calculated $M_1/M_{w(a)}$ *vs.* C curve which would fit the experimental data. However from this it could not be concluded that discrete self-association hypothesis failed to fit the data. In the above calculations all the association constants were assumed to be equal. It is possible that for a fixed value of n and BM_1 and varying K values, a curve may be

TABLE II: Determination of the Concentration of the Slow Peak from Area Analysis.

Total Protein Concn (g/l.)	Concn of Aggregable Material (g/l.)	Fraction of the Fast Peak	Concn of Fast Peak (g/l.)	Concn of Slow Peak (g/l.)
7.75	6.43	0.39	3.02	3.41
12.37	10.27	0.53	6.56	3.71
14.60	12.12	0.56	8.18	3.94
15.50	12.87	0.57	8.84	4.03
19.10	15.85	0.60	11.46	4.39
21.00	17.43	0.63	13.23	4.20
27.50	22.83	0.68	18.70	4.13
35.00	29.05	0.71	24.85	4.20
				Av 4.04 ± 0.23

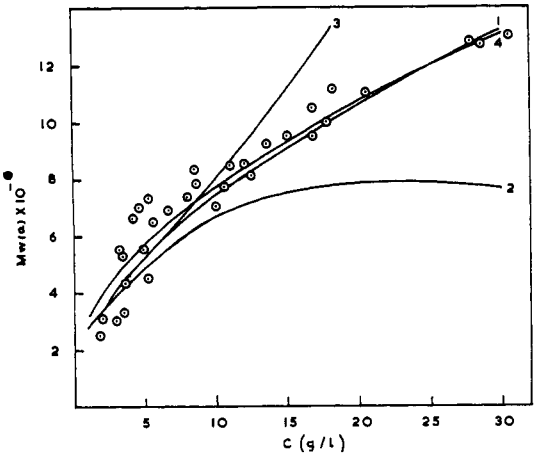

FIGURE 8: $M_{w(a)}$ *vs.* C curves calculated for nonideal self-association: (1) $BM_1 = 0$ (Adams' method); (2) $BM_1 = +0.005$ (Adams' method); (3) $BM_1 = -0.005$ (Adams' method); (4) $BM_1 = 0$ (method of Rossetti and Van Holde); (⊙) experimental data.

obtained which would fit the experimental data. It would indeed be surprising if such a fit was not obtained for some value of n, BM_1, and K.

The calculations described above showed that the indefinite self-association hypothesis (with $BM_1 = 0$) fitted the data the best compared to other hypotheses.

Analysis of Sedimentation Velocity Data. Since in sedimentation velocity experiments, bimodal patterns were obtained above a protein concentration of 5 g/l. the following calculations were possible.

(1) The dissociation constant derived from area analysis of the patterns was used to calculate $M_{w(a)}$ *vs.* C curve. Such calculated curves, as mentioned earlier, failed to fit the experimental data.

(2) With the equilibrium constant, the variation of the weight-average sedimentation coefficient of the whole boundary with protein concentration was calculated and compared with the experimental data. For this purpose eq 15 and 16 were used (Gilbert, 1963),

$$a_n = K_n a^n \tag{15}$$

$$\bar{S} = \frac{1 - g(C + \theta C)\sum_{n=1}^{\alpha} n a_n (S_n)_0}{\sum_{n=1}^{\alpha} n a_n} \tag{16}$$

where a_n is the concentration of n-mer (in mol/l.); K_n the *association* constant for the reaction monomer $\rightleftharpoons$ n-mer, $\bar{S}$, the weight-average sedimentation coefficient corresponding to the whole boundary; g, a constant relating sedimentation with concentration, C (g/l.); θ, the fraction of the total protein which did not aggregate; n, the value of n-mer; $(S_n)_0$, the sedimentation coefficient of the n-mer at $C = 0$.

(3) With the values of monomer concentration obtained from $M_{w(a)}$ *vs.* C data and for a monomer–n-mer model, $\bar{S}$ was calculated as a function of C and compared with the experimental data.

For the type of calculations involved under (2) or (3), g and $(S_n)_0$ are needed apart from a_n. The value of g was obtained from the experimental data. For the experimental patterns at various C, $\bar{S}$ was calculated by Goldberg's method (Goldberg, 1953). From the least-squares slope of $\bar{S}$ *vs.* C plot a value of $g = 0.005$ (g/l.)$^{-1}$ was obtained. A value of $\theta = 0.17$ was used since 17% autolysis product was produced. The $(S_1)_0$ of chymotrypsin monomer was taken as 2.4 S (Schwert and Kaufman, 1951); that of the n-mers was obtained with the relation

$$(S_n)_0 = (S_1)_0 (n)^{2/3} \tag{17}$$

Thus the particle was assumed to be an unhydrated sphere. No corrections were made for any deviation from this assumption. Such calculations based on the values of monomer concentration derived from $M_{w(a)}$ *vs.* C data gave a curve which did not fit the experimental data for any reasonable value of n (Figure 9).

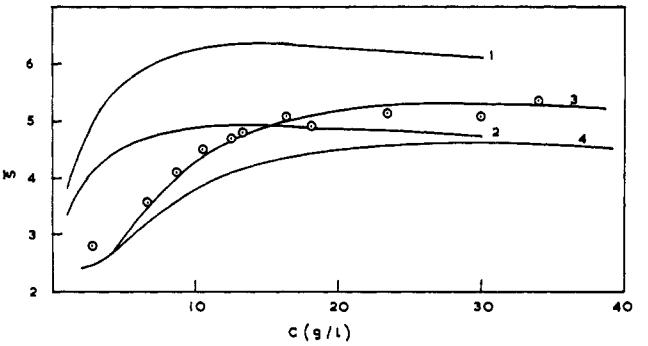

FIGURE 9: Calculated S *vs.* C curves for monomer–n-mer hypothesis: (1) for $n = 6$ and monomer concentration from $M_{w(a)}$ *vs.* C data; (2) for $n = 4$ and monomer concentration from $M_{w(a)}$ *vs.* C data; (3) for $n = 6$ and K from sedimentation velocity patterns; (4) for $n = 5$ and K from sedimentation velocity patterns; (⊙) experimental data.

With the dissociation constant derived from the area analysis, $\bar{S}$ was calculated as a function of C. Excellent agreement between the experimental data and the calculated curve was obtained for $n = 6$ (Figure 9). Thus a monomer–hexamer model seemed to fit the sedimentation data the best. The same conclusion was reached by Gilbert (1955, 1959) by an analysis of sedimentation velocity data of Massey *et al.* (1955) on the self-association of α-chymotrypsin in phosphate buffer of 7.9 and $\mu \sim 0.03$.

Thus there was a contradiction in the conclusions that could be drawn from the Archibald molecular weight and the sedimentation velocity data. From the former it could be concluded that indefinite self-association model with $BM_1 = 0$ was consistent with the data. On the other hand, the sedimentation velocity data would lead to a monomer–hexamer model. Although there was considerable scatter in $M_{w(a)}$ *vs.* C data, the fit between the experimental curve and that calculated for indefinite self-association model was satisfactory.

The Archibald molecular weights were determined at essentially atmospheric pressure; in sedimentation velocity experiments (done at 59,780 rpm) the system was subjected to considerable pressures, 100–500 atm. Kegeles *et al.* (1967) have shown by illustrative calculations that even a weak interaction may result in strong interaction in the ultracentrifuge cell at high speeds. They have recommended performing sedimentation velocity experiments at various speeds to detect the effect of pressure on the interaction. Using 1.5 and 2.0% protein solutions we have performed sedimentation velocity experiments at three speeds, namely 31,410, 47,660, and 59,780 rpm. The relevant sedimentation data are given in Table III.

The $s_{20,w}$ of the fast peak did not vary with speed; it was difficult to determine the $s_{20,w}$ value of the slow peak because of poor resolution, especially at the lowest speed. The weight-average $s_{20,w}$ of the entire peak, calculated from areas of the

TABLE III: $s_{20,w}$ of α-Chymotrypsin at Two Concentrations Determined at Several Speeds of Centrifugation.

Speed (rpm)	ω^2 [a] $= \left(\frac{2\pi \text{ rpm}}{60}\right)^2$	Protein Concn (g/l.)	$s_{20,w}$ Slow Peak	$s_{20,w}$ Fast Peak	% of Peak Slow Peak	% of Peak Fast Peak	Weight-Average $s_{20,w}$ [b]
31,410	1.0815×10^7	15		5.78			
		20		5.75			
47,660	2.4900×10^7	15	3.28	5.59	33 ± 2	67 ± 2	4.82
		20	3.20	5.57	35 ± 2	65 ± 2	4.74
59,780	3.9174×10^7	15	3.23	5.72	36 ± 2	64 ± 2	4.82
		20	3.15	5.95	37 ± 2	63 ± 2	4.91

[a] ω^2, a quantity proportional to the centrifugal field; rpm, revolutions per min. [b] From area analysis.

resolved peaks for slow and fast components, did not vary with speed; the proportion of the two peaks also did not vary with speed (Table III). Qualitatively, at lower speeds resolution was poor, perhaps, due to the effect of diffusion. These results appeared to indicate that self-association of α-chymotrypsin was not influenced by the speed of centrifugation.

We have indeed no explanation for the difference in conclusions that could be drawn from the Archibald molecular weight and sedimentation velocity experiments.

Effect of Autolysis Product(s) on Sedimentation Velocity Behavior of α-Chymotrypsin. It has been mentioned earlier that the addition of autolysis product(s) of α-chymotrypsin did not affect the self-association of the protein. The Archibald molecular weights, after correction, fitted the same $M_{w(a)}$ *vs.* C curve. It was, therefore, of interest to determine what effect the autolysis product(s) had on sedimentation velocity patterns. Bimodal peaks were obtained at concentrations much higher than in the absence of the autolysis product(s). Whereas in the absence of added autolysis product(s) bimodal peaks were obtained at protein concentrations higher than 5 g/l., such peaks were obtained at concentrations of ~11 g/l. and above, when autolysis product was added at 20% concentration. The $s_{20,w}$ of both the fast and the slow peak was lower (Figure 10). To determine the quantitative effect of added autolysis product on $s_{20,w}$ *vs.* C curve we calculated the curve for $n = 6$ and with $\theta = 0.32$. The calculated curve did not fit the experimental data. Here again there was an apparent contradiction between the Archibald molecular weights and the sedimentation velocity data. We nave indeed no explanation for this.

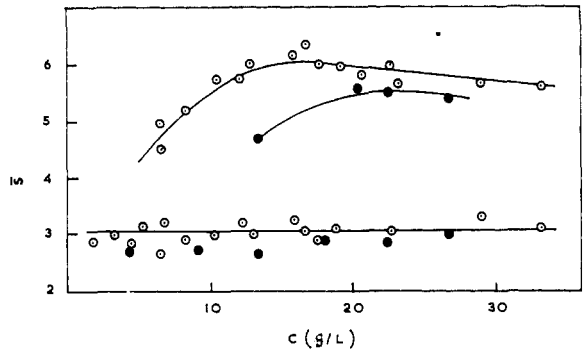

FIGURE 10: Sedimentation coefficient of fast and slow peaks as a function of protein concentrations: (⊙) in Tris buffer of pH 8.3 and $\mu = 0.05$; (●) in Tris buffer of pH 8.3 and $\mu = 0.05$ containing 20% autolysis product(s).

Acknowledgment

The authors are grateful to Mr. P. Jagan Mohan Reddy for help in writing the computer program and in computer calculations. The Model E ultracentrifuge used in this study was a gift from the Wellcome Trust, London.

References

Ackers, G. K., and Thompson, T. E. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 342.
Adams, Jr., E. T. (1965), *Biochemistry 4*, 1646.
Adams, Jr., E. T. (1967), Fractions No. 3, Beckman Instruments, Inc., Palo Alto, Calif., p 1.
Adams, Jr., E. T., and Filmer, D. L. (1966), *Biochemistry 5*, 2971.
Adams, Jr., E. T., and Fujita, H. (1963), *in* Ultracentrifuge Analysis in Theory and Experiment, Williams, J. W., Ed. New York, N. Y., Academic Press, p 119.
Adams, Jr., E. T., and Williams, J. W. (1964), *J. Amer. Chem. Soc. 86*, 3454.
Anderson, A. E., and Alberty, R. A. (1948), *J. Phys. Colloid Chem. 52*, 1345.
Egan, R., Michel, H. O., Schlueter, R., and Jandorf, B. J. (1957), *Arch. Biochem. Biophys. 66*, 366.
Gilbert, G. A. (1955), *Discuss. Faraday Soc. No. 20*, 68.
Gilbert, G. A. (1959), *Proc. Roy. Soc., Ser. A 250*, 377.
Gilbert, G. A. (1963), *Proc. Roy. Soc., Ser. A 276*, 354.
Goldberg, R. J. (1953), *J. Phys. Chem. 57*, 194.
Hartley, B. S., and Kaufman, D. L. (1966), *Biochem. J. 101*, 229.
Kegeles, G., Rhodes, L., and Bethune, J. L. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 45.
Massey, V., Harrington, W. F., and Hartley, B. S. (1955), *Discuss. Faraday Soc., No. 20*, 24.
McKenzie, H. A., Sawyer, W. H., and Smith, M. B. (1967), *Biochim. Biophys. Acta 147*, 73.
Morimoto, K., and Kegeles, G. (1967), *Biochemistry 6*, 3007.
Nichol, L. W., and Bethune, J. L. (1963), *Nature (London) 198*, 880.
Northrop, J. H., Kunitz, M., and Herriott, R. M. (1948), Crystalline Enzymes, Columbia University, New York, N. Y., p 118.
Rao, M. S. N. (1961), Symposium on Proteins, Chemical Research Committee and Society of Biological Chemists, India, p 1.
Rao, M. S. N., and Kegeles, G. (1958), *J. Amer. Chem. Soc. 80*, 5724.

Sarfare, P. S., Kegeles, G., and Kwon-Rhee, S. J. (1966), *Biochemistry 5*, 1389.
Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.
Schwert, G. W., and Kaufman, S. (1951), *J. Biol. Chem. 190*, 807.
Smith, E. L., and Brown, D. M. (1952), *J. Biol. Chem. 195*, 525.
Smith, E. L., Brown, D. M., and Laskowski, M. (1951), *J. Biol. Chem. 191*, 639.
Steiner, R. F. (1952), *Arch. Biochem. Biophys. 39*, 333.
Steiner, R. F. (1954), *Arch. Biochem. Biophys. 53*, 457.
Tinoco, I. (1957), *Arch. Biochem. Biophys. 68*, 367.
Van Holde, K. E., and Rossetti, G. P. (1967), *Biochemistry 6*, 2189.
Winzor, D. J., and Scheraga, H. A. (1963), *Biochemistry 2*, 1263.
Winzor, D. J., and Scheraga, H. A. (1964), *J. Phys. Chem. 68*, 338.