this technique to reacting systems already have been reported,[8,16] and the companion paper[17] describes a detailed study of such an application.

Acknowledgment.—This study has been supported by U. S. Public Health Service Research Grant RG-3449.

WORCESTER, MASS.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF CLARK UNIVERSITY]

An Ultracentrifuge Study of the Polymerization of α-Chymotrypsin

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The molecular weight of α-chymotrypsin has been determined as a function of protein concentration in phosphate buffer solution of *p*H 6.2 and ionic strength 0.2, by the Archibald ultracentrifuge method. From molecular weight measurements alone, the data can be interpreted within experimental error either by assuming monomers and trimers to be present or by assuming the simultaneous presence of monomers, dimers and trimers. The equilibrium constants and corresponding free energies of depolymerization have been calculated for both assumptions. In spite of the presence of polymers larger than the dimer, only one peak is observed in velocity ultracentrifugation. In conjunction with the recent theory of Gilbert for velocity ultracentrifugation of reacting systems, this fact would lend preference to the assumption that monomers, dimers and trimers are present together at equilibrium. A straightforward extension has been made of Gilbert's theory to the case where monomers, dimers and trimers are present simultaneously, and this predicts, moreover, only a single peak for velocity ultracentrifugation of such a system. It is therefore possible to deduce that under the conditions of these experiments, chymotrypsin is present as an equilibrium mixture containing monomers, dimers and trimers.

In a separate communication[2] we have justified the extension to the case of chemically reacting systems of the suggestions of Archibald[3] for the determination of molecular weights with the ultracentrifuge. The conditions under which such determinations may be expected to be strictly valid for reacting systems also have been indicated. This communication reports the results obtained by the application of these suggestions to a system containing α-chymotrypsin, which has been reported to undergo a concentration-dependent polymerization reaction.[3-7]

Experimental

The α-chymotrypsin was a Worthington Biochemical Corporation product, lot no. 577-82.

Electrophoretic mobility measurements were made with a Tiselius Electrophoresis Apparatus (Perkin–Elmer Model 38) fitted with a Longsworth scanning camera.[8] The isoelectric point of a 1% protein solution in phosphate buffers of ionic strength 0.2 was found to be *p*H 6.2. This value differs markedly from the reported values of 8.1[9] and 8.3.[10] The cited investigations were made in buffer solutions of univalent salts and of 0.1 ionic strength. The discrepancy can be explained on the basis that this protein binds phosphate ions strongly whereas it binds, perhaps, very little of the univalent buffer anions used by these investigators. That the phosphate ions are strongly bound by this protein is further suggested by the fact that the isoelectric point in phosphate buffer shifted to *p*H 6.9 when the ionic strength was decreased to 0.1; also when the experiments were repeated in acetate and glycine–sodium hydroxide buffer systems (ionic strength 0.1) the isoelectric *p*H was found to be 8.4 in good agreement with the reported values.[9,10] Molecular weight determinations were made with a Spinco Model E Ultracentrifuge, in a phosphate buffer solution of *p*H 6.2 and ionic strength 0.20 (0.029 *M* disodium hydrogen phosphate + 0.114 *M* sodium dihydrogen phosphate). The protein solutions were dialyzed in the cold, overnight against the buffer solution. At high protein concentrations it was observed that slight *p*H changes took place when the protein was dissolved in the buffer solution. Dialysis against a large volume of buffer solution is essential to restore the *p*H back to the original value.

The experimental technique and the method of molecular weight calculation described by Klainer and Kegeles[11,12] were followed, all experiments being performed with isoelectric protein to obviate the effects of possible non-ideality of the solutions. Previous work[11,12] had demonstrated no observable concentration-dependence of the molecular weight of simple proteins at their isoelectric points. The original protein concentrations, C_o in refractive index units were measured directly by using the boundary-forming cell.[13] At low and high protein concentrations, extrapolated values for C_o were used, extrapolation being done with a calibration curve of area *versus* concentration. For concentrations below 5 g. per liter, a cell with 30 mm. optical path was used and appropriate corrections to constant optical sensitivity were made in such cases. All other measurements were made with a standard 12 mm. cell. Carbon tetrachloride was used for the false bottom.

The individual experiments were done at various temperatures between 20 and 25°.

For the calculation of molecular weight a value of 0.73_6 was used for the partial specific volume of α-chymotrypsin.[5]

Absolute protein concentrations were determined by measuring the ultraviolet absorption at 282 mμ. The specific absorption coefficient determined with a portion dried to constant weight over phosphorus pentoxide was 2.07, in excellent agreement with the reported value[5] of 2.07_5.

Buffer salts were of reagent quality. *p*H measurements were made at 25° with a Beckman *p*H-meter, model G.

Results and Calculations

Figure 1 gives a plot of the weight-average molecular weight of α-chymotrypsin as a function of protein concentration. In this plot we have used the time-dependent values of C at the two

(1) Postdoctoral Fellow in Chemistry.

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(4) G. W. Schwert, *J. Biol. Chem.*, **179**, 655 (1949).

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(9) A. E. Anderson and R. A. Alberty, *J. Phys. Colloid Chem.*, **52**, 1345 (1948).

(10) V. Kubacki, K. D. Brown and M. Laskowski, *J. Biol. Chem.*, **180**, 73 (1949).

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

(12) S. M. Klainer and G. Kegeles, *Arch. Biochem. Biophys.*, **63**, 247 (1956).

(13) G. Kegeles, THIS JOURNAL, **74**, 5532 (1952).

menisci (air–solution and solution–carbon tetrachloride corresponding to the top and the bottom end, respectively, of the aqueous liquid column in the cell), rather than the values of original concentrations. Centrifugation usually was done for 45 minutes and pictures were taken at 15, 30 and 45 minutes after the attainment of the full operating speed. With the centrifugal fields employed in this study (3000–19,000 r.p.m.) and for such short intervals of time it was observed that marked changes of concentration with time did not occur at either meniscus. For any single experiment we have, therefore, averaged out the values of molecular weight and of concentration obtained at different intervals of time, the top and the bottom values being averaged separately. It is readily seen (Fig. 1) that the values from the top and the botton meniscus fit into a single curve.

Since in the case of other isoelectric proteins in this same range of concentration, no apparent concentration dependence of molecular weight is detectable,[12] it is justified to assume that in the present case the concentration dependence represents a real physical change in the system.

Thus by extrapolation of the curve to $C = 0$, the molecular weight of chymotrypsin monomer, the species favored by dilution, was obtained and was found to be 2.3×10^4. This is in good agreement with the value obtained by sedimentation-diffusion studies[14] and also with the value (at $C = 0$) obtained by the light-scattering method.[6] It may, however, be added that by the nature of the curve at low concentration (Fig. 1) very precise extrapolation is not possible.

By knowing the molecular weight of the monomer and applying the mass law equation, it is possible to calculate from values of weight-average molecular weight at various chymotrypsin concentrations the equilibrium constants for the association of monomers into polymers. However, before applying the mass law equation, it is necessary to know whether the system is truly in a condition of reversible equilibrium. For this purpose in two sets of experiments ultracentrifugation was carried out for a long time (2–18 hr.) so that considerable concentration changes took place at both menisci. The values of molecular weight at the top meniscus should decrease with time gradually due to polymer dissociation accompanying the decrease in concentration, and similarly the values at the bottom meniscus should increase with time. Reference to Fig. 1 shows that, in these prolonged experiments, the molecular weights do change with time in general qualitative agreement with this hypothesis. Thus the association of α-chymotrypsin seems to be truly reversible.

For the case of truly reversible equilibrium, the observed weight-average molecular weight when plotted against the slowly decreasing concentration at the upper meniscus, or against the slowly increasing concentration at the lower meniscus, should follow the same curve as that obtained from experiments of short duration at low speeds. However, if the chemical re-equilibration is not infinitely rapid, but is slow enough, the centrifugal field might be expected to deplete the solution of polymers at the upper meniscus, and to concentrate polymers at the lower meniscus, to an extent beyond that which would be expected at chemical equilibrium. Reference to Fig. 1 shows that in fact the values from the top meniscus gradually decrease below what they should be and those from the bottom increase above what they should be. Though the precision of our measurements is not high enough to derive any quantitative information, still the trend seems to be definite, indicating qualitatively that the rates of the association and dissociation reactions are not very large. The short-time experiments always were performed after leaving the solutions overnight, and numerous checks were obtained, giving some confidence that the circles in Fig. 1 indicating the short-time experiments represent equilibrium values.

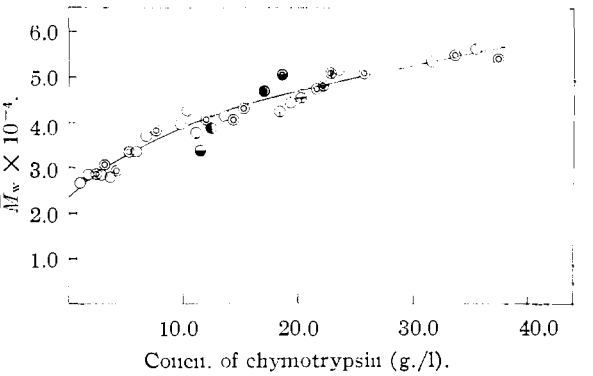

Fig. 1.—Experimental weight-average molecular weight *versus* concentration of chymotrypsin: ○, values from top meniscus; ⓞ, values from bottom meniscus; ⊕, 2 hr. values from prolonged experiments (starting concn. 20.3 g./l.); ⊙, 3 hr. values from prolonged experiments (starting concn. 20.3 g./l.); ◐, 6 hr. values from prolonged experiments (starting concn. 14.2 g./l.); ◒, 18 hr. values from prolonged experiments (starting concn. 14.2 g./l.).

We can write the equations for the over-all dissociation of an n-mer into n monomers as

$$n\text{-mer} \longrightarrow n \text{ monomers}; \quad K_n' = C_M^n/C_P \qquad (1)$$

where C_P and C_M are the weight-concentration of the n-mer and the monomer, respectively. If in the equilibrium system *only* monomers and n-mers are present, then the weight-average molecular weight $\bar{M}_w$ can be represented as

$$M_w = (C_M M_1) + C_P M_n/C \qquad (2)$$

where M_1 and M_n are the molecular weight of the monomer and n-mer, respectively, and C is the total weight-concentration. Since $C_P = (C - C_M)$ and $M_n = nM_1$, it can be shown that

$$K_n' = \left\{\frac{C}{(n-1)M_1}\right\}^{n-1} \frac{(nM_1 - \bar{M}_w)^n}{(\bar{M}_w - M_1)} \qquad (3)$$

Then K_n' is obtained from the slope of a plot of $(\bar{M}_w - M_1)/(nM_1 - \bar{M}_w)^n$ *versus* C^{n-1}.

First considering the case of dimerization ($n = 2$), the values of $(\bar{M}_w - M_1)/(2M_1 - \bar{M}_w)^2$ were plotted against values of C. The plot (Fig. 2) showed a pronounced upward curvature at high values of C. This fact, along with the observation that the experimentally determined weight-average

(14) N. Michael Green and H. Neurath, in "The Proteins," Ed. Neurath and Bailey, Vol. II, Part B, Acad. Press, Inc., New York, N. Y., 1954, pp. 1072, 1076–8.

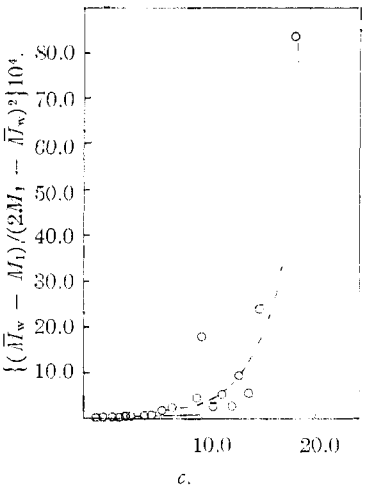

Fig. 2.—Plot of $(\bar{M}_w - M_1)/(2M_1 - \bar{M}_w)^2$ values *versus* C values; line near horizontal axis has the limiting slope.

molecular weights at high concentrations are greater than that of the dimer, suggests that polymerization proceeded beyond dimerization. For purposes of comparative analysis, a value of $K_2' = 2.07$ was obtained from the slope of the line drawn to fit the data up to a concentration of 13 g./liter and a molecular weight of 42,000. It would be incongruous to obtain a least square value from the data over the entire concentration range, because at $\bar{M}_w = 2M_1$ the slope would be infinite and at $\bar{M}_w > 2M_1$ it would have no physical significance. With this value of K_2', values of $\bar{M}_w$ as a function of C were calculated using equation 3. It can be seen from Fig. 4 that this calculated curve does not fit the data either in the low or in the high concentration range. Thus it seems reasonable to conclude that the dimerization hypothesis does not adequately explain the experimental data. However, if it is assumed that at *low* concentration dimerization is the predominant reaction then the constan for the dimerization reaction can be obtained from the limiting slope in Fig. 2, and this value of K_2' is 6.52. As is to be expected, the curve calculated with this value of K_2' fits the data well at low concentrations, but it completely fails to fit the data at high concentrations.

Similarly the case of trimerization ($n = 3$) was considered next. The plot of $(\bar{M}_w - M_1)/(3M_1 - \bar{M}_w)^3$ *vs.* C^2 (Fig. 3) does not show a pronounced curvature even at high concentration values, although there is considerable scatter in the points. Considering the extreme cases, we have drawn lines in Fig. 3 giving entire weight to points at low concentration or to points at high concentration (dashed lines in Fig. 3); the solid line has the slope obtained by least squaring the data over the entire concentration range. The values of $K_3' = 116.7$ and 45.0 were obtained from the lowest and the highest slopes, respectively; the value of K_3' from the least square slope is 56.2. With these various values of K_3', $\bar{M}_w$ as a function of C was calculated with equation 3. These calculated curves are given in Fig. 4. In view of the scatter in the experimental points it is reasonable to conclude that even the curves calculated with the extreme values of

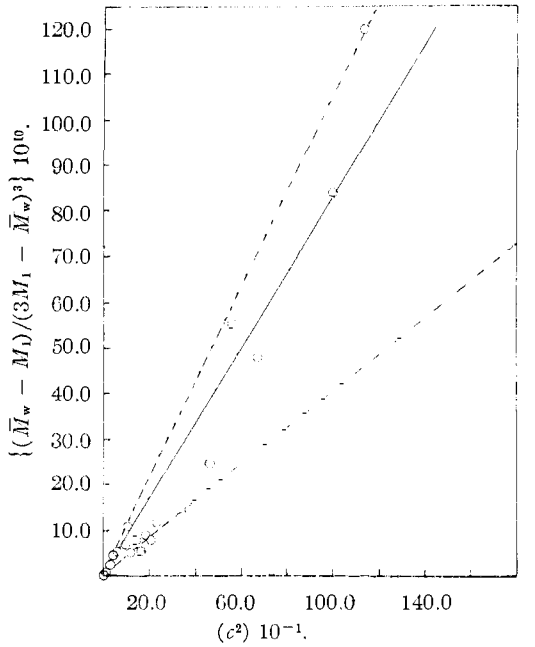

Fig. 3.—Plot of $(\bar{M}_w - M_1)/(3M_1 - \bar{M}_w)^3$ values *versus* C^2 values: ·····, lines drawn giving entire weight to data at low concentration *or* high concentration only; ———, line has the least square slope.

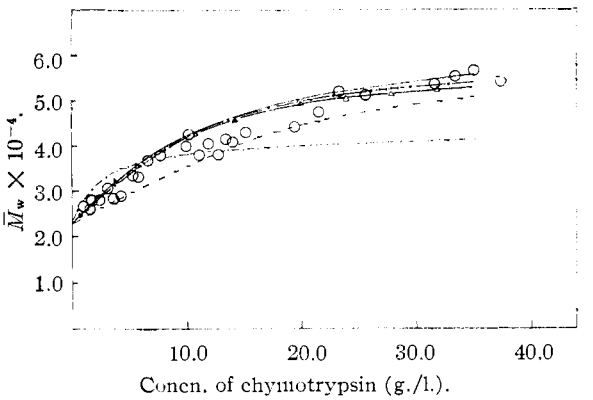

Fig. 4.—Experimental points and theoretical curves for weight-average molecular weight *versus* concentration of chymotrypsin: -·-·-·, calculated for dimerization only $K_2' = 2.07$; ·····, calculated for trimerization only $K_3' = 116.7$; —·—, calculated for trimerization only $K_3' = 56.2$; ———, calculated for trimerization only $K_3' = 45.0$; —△—, calculated for dimerization and trimerization, $K_2 = 11.1$ and $K_3 = 4.5$; —○—, experimental points.

K_3' fit the data fairly well. From the above analysis a possible picture of the polymerization equilibrium is that only monomers and trimers are present in the mixture.

Both from the point of view of kinetics of the polymerization reaction and from the fact that equation 3 written for dimerization holds good at low concentration, it may also be postulated that the equilibrium mixture contains all the three species, monomers, dimers and trimers. For the case when all the species are present (monomer to n-mer) the weight-average molecular weight $\bar{M}_w$ can be represented[15] in the present notation by

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

$$M_w = \frac{C_M M_1 + 2(1/K_2)C_M^2 M_1 + 3(1/K_2K_3)C_M^3 M_1 + \ldots + n(1/K_2K_3 \ldots K_n)C_M M_1}{C} \quad (4)$$

where

$$K_2 = \frac{C_M^2}{C_D},\ K_3 = \frac{C_D C_M}{C_T},\ \ldots K_n = \frac{C_{n-1}C_M}{C_n}$$

and C_M, C_D, C_T ... C_n are the weight concentration of the monomer, dimer, trimer and n-mer, respectively. If we define x such that $C_M = xC$ then equation 4 can be rewritten as

$$C\cdot\bar{M}_w = (xC)M_1 + 2(1/K_2)(xC)^2M_1 + 3(1/K_2K_3)(xC)^3\cdot M_1 + \ldots + n(1/K_2K_3 \ldots K_n)(xC)^nM_1 \quad (5)$$

Following Steiner's[15] treatment, we can write

$$\ln x = \int_0^C \{(\alpha^{-1} - 1)/C\}dC$$

where $\alpha = \bar{M}_w/M_1$. The values of x as a function of C can be obtained by means of graphical integration. With these values, we can plot $\{\bar{M}_w/xM_1 - 1\}xC$ as a function of xC. From the intercept and the slope, the values of K_2 and K_3, respectively, can be calculated. Any upward curvature in the plot would suggest the existence of polymers higher than trimers.

The plot showed a slight upward curvature but the precision of our data is not high enough to place any emphasis on this curvature. By least squaring the data values of $K_2 = 11.1$ and $K_3 = 4.5$ were obtained.

The value of $K_2 = 11.1$ obtained by this method of calculation compares directly with the value of $K_2' = 6.52$ obtained above by considering the process at low concentration as comprising the dissociation of dimers only, according to equation 3. The value for K_3 obtained by Steiner's method is 4.5 and since $K_3 = K_3'/K_2'$, the value of K_3 calculated from the values of K_2' and K_3' obtained with equation 3 is 8.62. The agreement in the values of K_2 and K_3 obtained by two methods can be considered fairly satisfactory.

With the values of $K_2 = 11.1$ and $K_3 = 4.5$ in equation 5, $\bar{M}_w$ was calculated as a function of C. It is seen from Fig. 4 that this curve is essentially the same as the one calculated by least squaring the data to equation 3 for the case of trimerization alone. Thus the interpretation that all the three species—monomer, dimers and trimers—are present in the equilibrium mixture seems to be as valid as the one that only monomers and trimers are present.

If in equation 4 the concentration is expressed in moles per liter and the successive dissociation constants are designated k_2, k_3, etc., it readily can be shown that

$$k_2 = K_2(2/M_1);\ k_3 = K_3(3/2M_1), \text{ etc.} \quad (6)$$

The values of k_2 and k_3 are 0.96×10^{-3} and 0.29×10^{-3}, respectively. Thus, on this scale, the constant for the dissociation of dimers into monomers is nearly three times greater than the constant for the dissociation of trimers into dimers and monomers.

Discussion

To obtain some idea of the relative stability of dimers and trimers, assuming that monomers, dimers and trimers are all present, it is desirable to examine the constants K_2 and K_3, on the grams/liter concentration scale, and the corresponding standard free energies for dissociation. This concentration scale is useful for such a comparison, since the standard state reaction corresponds to polymer, at a concentration of one gram per liter and unit activity coefficient, dissociating into products, each also at one gram per liter concentration and unit activity coefficient. These concentrations and activity coefficients correspond to experimentally realizable physical conditions, whereas it is quickly seen that the physical conditions corresponding to the standard state on the mole/liter scale are completely incongruous, because of the very large molecular weight of protein molecules. Failure to consider the physical realizability of the standard state can lead to confused deductions from the experimentally derived thermodynamic data.

In order to eliminate the standard free energy of monomer, the reaction

$$\text{dimer} \longrightarrow 2 \text{ monomers};\ \Delta F_2^0 = -RT \ln K_2 = -1411 \text{ cal./mole dimer} \quad (7)$$

is multiplied by $^1/_2$ and subtracted from the reaction

$$\text{trimer} \longrightarrow \text{monomer} + \text{dimer};\ \Delta F_3^0 = -RT \ln K_3 = -881 \text{ cal./mole trimer} \quad (8)$$

giving the reaction

$$\text{trimer} \longrightarrow 3/2 \text{ dimer};\ \Delta F^0 = \Delta F_3^0 - {}^1/_2\Delta F_2^0 = -176 \text{ cal./mole trimer} \quad (9)$$

This implies that a solution containing 1 g./liter of chymotrypsin trimer would tend to dissociate spontaneously to chymotrypsin dimer, at a concentration of 1 g./liter, with a free energy change of $\Delta F^0 = -176$ cal./mole of chymotrypsin trimer. Equation 7 also implies that at the same 1 gram/liter concentration level of protein species, dimers would dissociate spontaneously into monomers with a free energy change of −1411 cal./mole of chymotrypsin dimer.

It was desired to compare the moving boundary sedimentation behavior of this chemically reacting system with its predicted behavior from the recent theories of Gilbert[16] and of Gilbert and Jenkins[17] once a reasonable idea of the species present had been obtained by the presently employed non-hydrodynamic method. Three moving boundary ultracentrifuge experiments were performed at 59,780 r.p.m. in phosphate buffer of pH 6.2 and ionic strength 0.2. The chymotrypsin concentrations were 15.0, 18.9 and 36.0 grams per liter. In each case a single fairly symmetrical peak was observed. These observations are in qualitative agreement with the results of Schwert[4] under roughly comparable conditions. Reference to Table I provides an idea of the concentration of each species present at a total chymotrypsin concentration of 18.9 g./liter; the calculation has been made under two separate assumptions, first that only monomers and trimers

(16) G. A. Gilbert, *Disc. Faraday Soc.*, **20**, 68 (1955).
(17) G. A. Gilbert and R. C. Ll. Jenkins, *Nature*, **177**, 853 (1956).

are present (column 2) and second, that monomers, dimers and trimers are all present (column 3).

TABLE I

SPECIES CONCENTRATIONS IN CHYMOTRYPSIN (G./L.)

Species concn., g./l.	Eq. 3 (trimerization) $K_3' = 56.2$	Eq. 4 $K_2 = 11.1$; $K_3 = 4.5$
C_{Total}	18.90	18.90
$C_{Monomer}$	8.24	7.16
C_{Dimer}	...	4.50
C_{Trimer}	10.66	7.24

It is clear from Gilbert's treatment of the case of infinitely rapid re-equilibration in a monomer–trimer reaction that two peaks should be expected in velocity ultracentrifugation, provided that diffusion can be neglected and dimers are not also present. From the treatment of Gilbert and Jenkins for a rapid reaction $A + B \rightleftarrows C$ it might perhaps be expected that three peaks would appear in velocity ultracentrifugation if the predominant reaction is the dissociation of trimers to dimers and monomers, all three species being present. However, the case treated by these authors does not really apply to the chymotrypsin system, since this treatment does not provide for interconversion of the slowest species B into other species, once separation of this single species has been completed. On the other hand, if the original theory[16] of Gilbert is taken as correct, then the information that monomers and trimers are necessarily present, as judged from Fig. 2, and the information that only one peak is observed in velocity ultracentrifugation must lead to the conclusion that dimers are also present at equilibrium. For otherwise Gilbert's theory would require two peaks in velocity ultracentrifugation, which is contrary to experimental observation. Furthermore, the assumption of finite dissociation and association rates could only be expected to lead to better resolution in the velocity ultracentrifuge experiments.

According to Gilbert,[16] in the theory of boundary shapes in velocity ultracentrifugation of a monomer–polymer system which neglects diffusion, "no new principle is involved when a group of complexes (each of different n) is present, but the algebra is much more complicated." Fortunately, a straightforward extension of Gilbert's original theory to the case of coexisting monomers, dimers and trimers has been found to lead to a closed solution for the gradient of total concentration. This extension is indicated briefly here, reference to Gilbert's paper[16] being recommended for a more detailed discussion. The system is assumed to react so rapidly that it is always at chemical equilibrium. The cell is assumed to be rectangular. The relative amounts of monomer, dimer and trimer are then related through equation 1. If a frame of reference is chosen which is stationary with respect to the monomer, and the velocities of dimer and trimer, v_D and v_T, are taken as constant, the following equation applies

$$\left\{\frac{\partial(C_M + C_D + C_T)}{\partial t}\right\}_x + v_D\left(\frac{\partial C_D}{\partial x}\right)_t + v_T\left(\frac{\partial C_T}{\partial x}\right)_t = 0 \quad (10)$$

The relative velocity δ is defined by

$$\left(\frac{\partial x}{\partial t}\right)_C \frac{1}{v_T} \equiv \delta = \frac{x - x_0}{v_T t} - \frac{v_M}{v_T} \quad (11)$$

In these equations t is the time since the start of the experiment, and x is the distance from the center of rotation, x_0 being the top surface of the liquid column. By taking advantage of the mathematical relationships between partial derivatives in equation 10 and applying equation 1 to calculate concentration of dimer and trimer in terms of the concentration of monomer, it is found that the maximum permissible value of δ is given by

$$\delta = \frac{\frac{v_D}{v_T}\frac{2}{K_2'}C_M + \frac{3}{K_3'}C_M^2}{1 + \frac{2}{K_2'}C_M + \frac{3}{K_3'}C_M^2} \quad (12)$$

where C_M at any given total concentration of chymotrypsin is obtained by Steiner's procedure, as outlined above. The total concentration of protein is given by

$$C = C_M + C_D + C_T = C_M + \frac{C_M^2}{K_2'} + \frac{C_M^3}{K_3'} \quad (13)$$

and differentiation then indicates that

$$\left(\frac{\partial C}{\partial x}\right)_t = \frac{C_M^2}{v_T t}\,\frac{\left(\frac{v_D}{v_T}\frac{2}{K_2'} + \frac{3}{K_3'}C_M\right)^2}{\delta^2\left\{\left(\frac{v_D}{v_T}\frac{2}{K_2'} + \frac{6}{K_3'}C_M\right) - \delta\left(\frac{2}{K_2'} + \frac{6}{K_3'}C_M\right)\right\}} \quad (14)$$

From equation 12, C_M is found in terms of δ, and insertion of this value into equation 14 results finally in

$$\left(\frac{\partial C}{\partial x}\right)_t = \frac{K_3'^2}{9\delta^2(1-\delta)^4}\frac{1}{v_T t}\,\frac{\left(\frac{v_D/v_T - \delta}{K_2'} - D(\delta)\right)^2\frac{1}{K_2'}\left[(1-2\delta)\frac{v_D}{v_T} + \delta\right] + D(\delta)\left(\frac{v_D/v_T - \delta}{K_2'} - D(\delta)\right)^2}{2D(\delta)} \quad (15)$$

where

$$D(\delta) = \left[\left(\frac{v_D/v_T - \delta}{K_2'}\right)^2 + \frac{3\delta(1-\delta)}{K_3'}\right]^{1/2} \quad (16)$$

It is noted from equation 12 that $C_M = 0$ when $\delta = 0$, and by evaluation of the resulting intermediate form in equations 14 and 15 it is found that

$$\lim_{\delta \to 0}\left(\frac{\partial C}{\partial x}\right)_t = \frac{K_2'}{2v_D t} \quad (17)$$

The values of v_D and v_T which are required to evaluate equation 15 can be calculated from the sedimentation coefficients of the dimer and trimer, respectively. For the purposes of these calculations, the sedimentation coefficients of the chymotrypsin

dimer and trimer were computed using the following relationship[18] and using a value of 2.4 S for the sedimentation coefficient of the monomer,[5]

$$S_D/S_M = \{2M_1/M_1\}^{2/3} = 2^{2/3};$$

$$S_T/S_M = \{3M_1/M_1\}^{2/3} = 3^{2/3}$$

where S_M, S_D and S_T are the sedimentation coefficients of monomer, dimer and trimer, respectively, and M_1 is the monomer molecular weight. In using the above relationship, the basic assumption is that these molecules are spherical in shape. Since these values are only for purposes of sample computations of the gradient curves, there should not be much objection to the procedure adopted for getting S_D and S_T values. The calculated S_D and S_T values are 3.80 and 4.99S, respectively.

Using the values of $v_D/v_T = 0.762$, $K_2' = 11.1$, $K_3' = 50.0$ in equation 15, the dC/dx values as a function of δ were calculated for a total concentration of 18.9 g./l. and for $v_T t = 0.9159$ cm. The monomer concentration that corresponds to this total concentration is 7.16 g./l. (equation 4), and this fixes the maximum value of δ at 0.756 (equation 12). In Fig. 5B, dC/dx values are plotted as a function of δ. It is seen that the schlieren pattern consists of a single peak. Similar calculations, for the same total concentration and $v_T t$ value, were made using the equations given by Gilbert[16] for the case when only monomers and trimers are present. The result is a double peak (Fig. 5A) with a valley at $\delta = 0.167$.

Comparison with an actual sedimentation velocity pattern, for the same total concentration of 18.9 g./l. (Fig. 5C), leads to the conclusion that, under the experimental conditions, chymotrypsin exists as an equilibrium mixture of monomers, dimers and trimers. It has been pointed out earlier that from molecular weight data alone both the hypotheses—that monomers and trimers only are present or that all the three species are present—were equally valid. Thus with the aid of Gilbert's theory and values of the weight-average molecular weights, the sedimentation velocity experiments, at any rate for this system, seem to give a decisive answer.

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(18) Svedberg and Pedersen, "The Ultracentrifuge," Oxford University Press, 1940, p. 10.

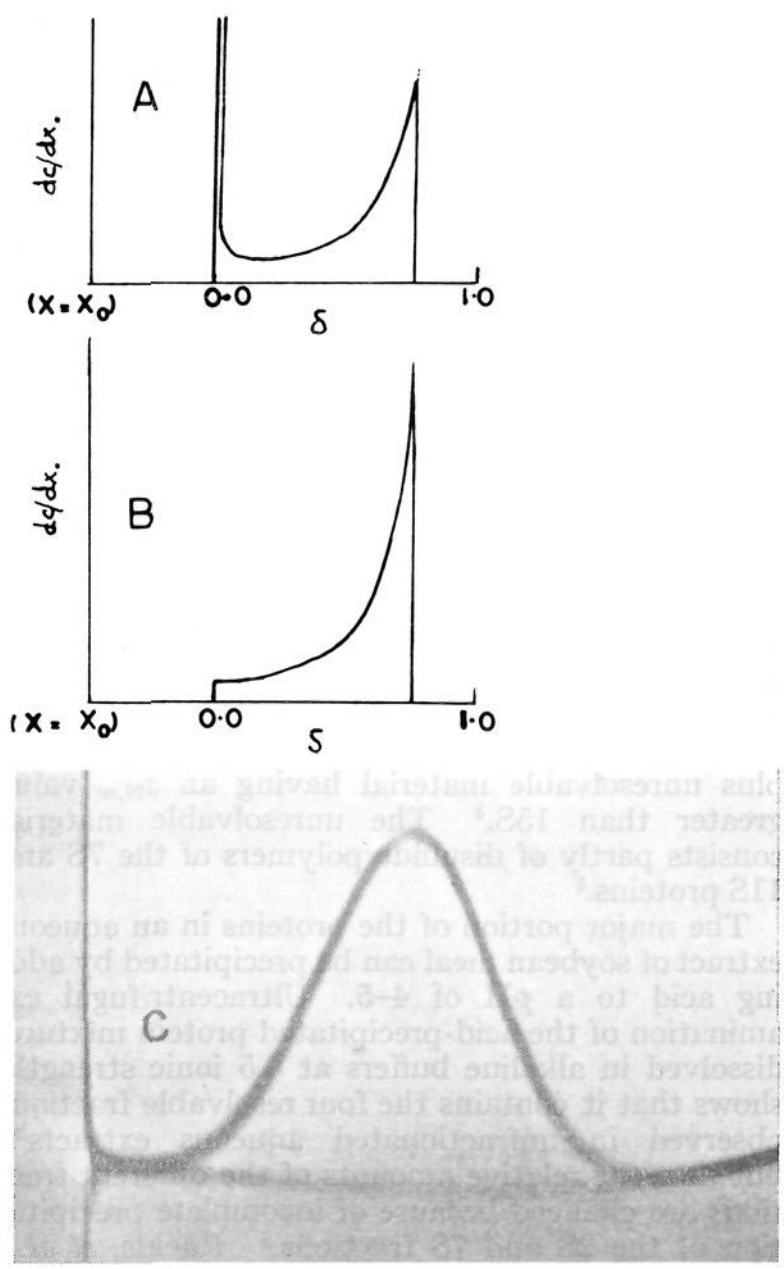

Fig. 5.—Theoretical and experimental sedimentation velocity patterns: A, theoretical pattern for the case of equilibrium mixture of chymotrypsin monomers and trimers, for a total concentration of 18.9 g./l.; B, theoretical pattern for the case of equilibrium mixture of chymotrypsin monomers, dimers and trimers for a total concentration of 18.9 g./l. The position $x = x_0$ corresponds to a value of $\delta = -v_M/v_T$; C, experimental sedimentation velocity pattern of chymotrypsin. Protein concentration = 18.9 g./l.; centrifugation for 120 min. at 59,780 r.p.m. Sedimentation proceeds from left to right.

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