Self-Association of Muramidase (Lysozyme) in Solution at 25°, pH 7.0, and $I = 0.20$*

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ABSTRACT: When dissolved in sodium cacodylate buffer of ionic strength 0.20 and pH 7.0, and at 25°, it has been found that muramidase is subject to a moderate degree of self-association. The apparent weight-average molecular weight of the protein as a function of its concentration has been determined by using the low-speed sedimentation equilibrium experiment. This information has been subjected to numerical analyses in the attempt to provide a detailed description of the self-association type. Two mathematical models have been considered, the monomer–dimer and isodesmic mechanisms. It has been found that up to 2.2 g/dl, the values of $M_{w(c)}^{app}$ which have been found are about equally well suited by either mechanism.

In spite of this situation, it does appear that K_2 for the monomer–dimer model and $2k$ for the isodesmic one are approximately equal, suggesting that it may be possible to proceed with calculations for the thermodynamic quantities which characterize the dimerization step of the reaction.

Since there are simple mathematical methods for analyzing macromolecular self-associations in solution [for example, see Adams (1967) and Elias and Bareiss (1967)], it is tempting to proceed toward interpretation of the molecular weight data in terms of thermodynamic quantities. Such an effort would appear to be particularly fruitful for proteins whose structures are well established, since data might then be interpreted on the basis of known molecular geometry. Hen's egg white muramidase would appear to be an excellent protein to examine, since it has been analyzed by X-ray crystallography (Blake *et al.*, 1965), and its self-association has been studied (Sophianopoulos and Van Holde, 1964; Adams and Filmer, 1966; Bruzzesi *et al.*, 1965). Because the data of Sophianopoulos and Van Holde were interpreted by assuming that the solutions were ideal and because results from the two more recent investigations are somewhat imprecise, we here present additional apparent weight-average molecular weight data for the self-associating muramidase molecule, this time in NaCl–cacodylate buffer, pH 7.0, ionic strength $I = 0.20$, and at 25°. With the improved data, we have endeavored to assign a model to the self-association as a preliminary step in a more extensive thermodynamic analysis. In their interpretation, it is found that there are ambiguities associated with such an assignment which is based on apparent weight-average molecular weight data alone.

Theory

Our information consisted entirely of refractometric data from the ultracentrifuge, which were processed to yield first c *vs.* r data, and then $M_{w(c)}^{app}$, the apparent weight-average molecular weight, as a function of concentration. In the absence of independent measures of self-association, such as transport experiments, the assignment of a model to apparent molecular weight data is based upon curve fitting. The criteria for "accepting" a model are that the data be well represented over an extended concentration range, and that the solution nonideality be described by physically reasonable parameters.

It is assumed that the activity coefficient of the ith species, y_i, may be represented as follows (Adams and Fujita, 1963; Adams and Williams, 1964)

$$\ln (y_i) = iM_1Bc + \ldots \quad (1)$$

where i is the degree of association of the ith species, M_1 is the molecular weight of the monomer, B is the second virial coefficient, which has been assumed to be identical for all species, and c is the concentration in g/dl. At sufficiently high concentrations, eq 1 may be expected to be inadequate for the description of the solution nonideality, but there is no simple, general way of predicting at just what point a third virial coefficient will be required. For proteins, both excluded volume considerations and the Donnan effect tend to make B positive, so that any models which require use of negative values for B may be considered to be suspect. Any negative contributions to the second virial coefficient may instead be accounted for by equilibrium constants describing a self-association. Thus, any increase in apparent molecular weight is attributed to a self-association reaction, for which a set of equilibrium constants K_{i+1} is introduced to describe the formation of each species larger than monomer. If c_i is the concentration in g/dl of species i, and c_1 is the concentration of monomer, then

$$c_{i+1} = K_{i+1}c_ic_1 \quad (2)$$

Because we have only one kind of data at our disposal, we shall adopt the practice of using the smallest number of K's which will provide for adequate representation of the data, which means that the choice of the model will depend in

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part on the precision of the experiment. Because the apparent weight-average molecular weight data (Figure 1) indicate that the tendency for muramidase to self-associate is small under the present experimental conditions and because the apparent weight-average molecular weight is still well below 28,600 g/mole at the highest concentration investigated, it seemed likely that a monomer–dimer equilibrium with a small positive BM_1 might provide for adequate representation of the data. In this event, it might be sufficient to consider only the class of two-parameter representations, which includes principally the nonideal monomer–*n*-mer self-associations and the nonideal indefinite (isodesmic) self-associations.

The indefinite self-association is described by the equation

$$\frac{R_a^2}{(1 - BM_1R_ac)^2} - 1 = 4kc \qquad (3)$$

where R_a is the ratio $M_{w(c)}^{app}/M_1$, M_1 is the monomer weight, c is the concentration in g/dl, and k is an equilibrium constant on the mass-based concentration scale so defined that the corresponding equilibrium constant on the mole-based scale is the same for each species formed (Van Holde and Rossetti, 1967). Methods for treating data by means of eq 3 have been described by Van Holde and Rossetti (1967) and by Van Holde *et al.* (1969).

Of the remaining two-parameter models, the nonideal monomer–dimer mechanism seemed to be most appropriate. This model may be treated by general methods which have been described in the report of Adams and Filmer (1966), whose formulation requires successive approximation procedures for solving the appropriate equations. However, the method which they have outlined does not provide a simple means of fitting the data to the model with simultaneous minimization of residuals over the entire concentration range. Here we present a different approach, one which allows the determination of the two parameters of the monomer–dimer model by an iterative procedure based upon a least-squares fit of appropriate quantities to a linear equation.

For a system containing monomer in rapid chemical equilibrium with its dimer, the apparent weight-average molecular weight of the solute may be expressed as an explicit function of concentration in the following way (Adams and Fujita, 1963)

$$2(M_1/M_{w(c)}^{app}) - 1 = (1 + 4K_2c)^{-1/2} + 2BM_1c \qquad (4)$$

Because the concentration c is given in g/dl, the equilibrium constant K_2 and the product BM_1 both have units of dl/g. For notational simplicity, the following definitions are introduced

$$\begin{aligned} a &= 4K_2 \\ b &= BM_1 \\ F(c) &= 2(M_1/M_{w(c)}^{app}) - 1 \end{aligned} \qquad (5)$$

Equation 4 then becomes

$$F(c) = (1 + ac)^{-1/2} + 2bc \qquad (6)$$

The term $(1 + ac)^{-1/2}$ may cause $F(c)$ to vary rapidly with concentration as c approaches zero; therefore, to generate a more slowly varying function, it is necessary to eliminate the radical. The following definition is made

$$G(c) = \int_0^c F(c')dc' = \frac{2}{a}[(1 + ac)^{1/2} - 1] + bc^2 \qquad (7)$$

Then eq 6 and 7 may be combined to eliminate the term $(1 + ac)^{1/2}$, with the following result

$$G(c) - bc^2 = \frac{2}{a}[F(c) - 2bc]^{-1} - \frac{2}{a} \qquad (8)$$

This may be rearranged to yield

$$\frac{F(c)G(c)}{1 - F(c)} = \frac{2}{a} + b\frac{c^2F(c) + 2cG(c)}{1 - F(c)} - 2bc\frac{(bc^2 - 2/a)}{1 - F(c)} \qquad (9)$$

which is written

$$U = 2/a + bV - W \qquad (10)$$

The significance of each of the quantities U, V, and W is readily obtained from direct comparison of eq 9 and 10. The quantities U and V can be calculated from the apparent weight-average molecular weight data. If W is negligible in comparison with U, a plot of U against V yields a straight line, from which the parameters $2/a$ and b can be determined as the ordinate intercept and slope. If W is not negligible, but still is sufficiently small, eq 10 can be solved by an iterative process in which the values of W are estimated from previously obtained approximations for $2/a$ and for b. Because of the division by $1 - F(c)$ indicated in eq 9, the use of this equation is restricted to those values of c for which $F(c)$ is less than unity. Convergence and further practical considerations are discussed elsewhere (Deonier, 1970).

An alternative method for determining the parameters for the monomer–dimer model is analogous to the Van Holde–Rossetti method for the indefinite self-association. From eq 4 one may write

$$\frac{R_a^2}{[2(1 - BM_1R_ac) - R_a]^2} - 1 = 4K_2c \qquad (11)$$

where the significance of the various quantities has already been given. After dividing both sides of eq 11 by c, the procedure would then be to fit the left-hand side of the resulting equation to a linear equation in c by the least-squares method and to assign arbitrary values of BM_1 until a value is found for which the linear equation has a slope of zero.

Methods

Preparation of Protein. Hen's egg white muramidase (EC 3.2.1.17) was crystallized from egg white with 5% NaCl (4°, pH 10) by the method of Alderton and Fevold (1946). After three crystallizations, the protein was dialyzed against deionized H_2O for 24 hr and then lyophilized. Additional purification was accomplished by ion-exchange chromatography, using Bio-Rex 70 cation-exchange resin and the conditions described by Stevens and Bergstrom (1967). The elution patterns for this muramidase preparation showed all the major

features found by these workers for Pentex 3× crystallized lysozyme. The central fractions of the main peak were pooled and dialyzed against H_2O for 24–60 hr. The solution was then lyophilized and the muramidase powder was stored at 0° until use.

Buffer Solutions. Muramidase association was studied at pH 7.0, in 0.20 ionic strength NaCl–cacodylate buffer at 25°. Buffers were prepared with the following composition: 0.14 M NaCl–0.06 M NaOH–9.4 g/l. of cacodylic acid. Deionized water was used for all preparations. A Beckman Model G pH meter yielded pH readings, which for all buffer preparations were 7.00 ± 0.03 at 20°. Buffer densities at 25° (ρ_0) were measured with the use of 20-ml Ostwald-Sprengel pycnometers. All required weighings were performed with a Mettler B5 balance. Because the amount of cacodylic acid required to adjust the pH varied slightly from preparation to preparation, the densities of the different buffer preparations varied by 0.0001 g/cm³ from the average value, which for three buffer preparations was 1.0093 g/cm³ at 25°. This figure, which has been corrected for air buoyancy, was used for ρ_0 in the factor $(1 - \bar{v}\rho_0)$.

Protein Solutions. Solutions of muramidase were prepared by sprinkling the lyophilized powder over 2–5 ml of the cacodylate buffer. The powder dissolved rapidly, but there remained a slight turbidity. Solutions were introduced into 0.375-in. Visking casing which had been boiled in deionized water and then soaked in the buffer for several hours. Dialysis proceeded for 30–60 hr at 4° with four to six buffer changes in 250-ml increments. After dialysis, any turbidity was removed either by centrifugation or by filtration through a 0.45-μ Millipore filter. The solution and a portion of the final dialysate were stored in glass containers at 4° until use. In nearly all cases the solutions were used within a week after dialysis.

Concentrations were determined spectrophotometrically with a Beckman DB spectrophotometer. The muramidase absorptivity for a 1% solution in a 1-cm cell was taken to be 26.35 dl/g-cm at 280 mμ (Sophianopoulos *et al.*, 1962). To obtain precise absorbance readings gravimetric dilutions with water were performed by using the Mettler B5 balance. For calculating the dilution factor the protein solution density ρ was calculated by use of the expression $\rho = \rho_0 + (1 - \bar{v}\rho_0) \times c/100$ in which ρ_0 is again the solvent density, $\bar{v}$ is the partial specific volume of the protein, and c is its concentration in g/dl. No buoyancy corrections were applied in the calculation of the dilution factors. At least three concentration determinations were performed for each protein sample, with a relative precision of 0.5%. Muramidase solutions having concentrations lower than those of the stock solutions were obtained by diluting the stocks with dialysate. Concentrations of these solutions were determined spectrophotometrically in the manner described above.

Subsidiary Measurements. The specific refraction increment $(dn/dc)_\mu$ at 546 mμ, 25°, and the apparent specific volume of muramidase at 25° in the cacodylate buffer system were determined by using three solutions with the following protein concentrations: 0.3565 g/dl, 0.4798 g/dl, and 0.5665 g/dl. Each sample had been dialyzed at 4° against buffers of identical composition. All measurements were performed by using the dialysate as the solvent. Concentrations were measured spectrophotometrically, using a Gilford Model 222 photometer coupled to a Beckman Model DUR quartz monochromator. No dilution of samples was required for these measurements since a 1-mm silica cuvet was used. All absorbance readings were corrected to 1 cm by using the measured thickness of the cuvet along the optic axis. Repeated measurements with the same sample agreed to within 0.001 to 0.002 absorbance unit. Concentrations were calculated by using the absorptivity given by Sophianopoulos *et al.* (1962).

The specific refraction increment at 546 mμ and 25° in cacodylate buffer was measured with a differential refractometer which had been calibrated with sucrose (NBS standard sample 17) dissolved in triple-distilled water. The slit image displacement was directly proportional to the difference in refractive index between the dialysate and the solution over the refractive index range covered by the three samples. Although the literature values for (dn/dc) at 546 mμ are close to 0.001888 dl/g (Halwer *et al.*, 1951; Bruzzesi *et al.*, 1965), the value found in this work was 0.001850 dl/g.

The apparent specific volume of muramidase for the NaCl–cacodylate buffer system was computed from the densities of the three protein solutions with the aid of the equation

$$(\partial\rho/\partial C)_\mu = (1 - \phi'\rho_0) \tag{12}$$

where $(\partial\rho/\partial C)_\mu$ is the change in density of the solution with respect to protein concentration (in g/cm³) at constant chemical potential of diffusible components, ρ_0 is the density of the dialysate, and ϕ' is the apparent specific volume of the protein, which is assumed to be equivalent to its partial specific volume. The densities of the three protein solutions and of the dialysates were measured with pycnometers fashioned from erlenmeyer flasks with capillary necks and equipped with ground-glass caps. The pycnometer volumes (approximately 29 cm³) were calibrated with water from a Barnstead still. Dialysate densities were measured by using different samples for each determination, but because of the limited supply of protein solution, duplicate measurements of the solution densities were performed by using the same portion of solution. Both the dialysate and the protein solutions had been stored at 4°; consequently, it was essential to degas them with a syringe in order to obtain reproducible densities. All masses were corrected for air buoyancy. The densities as a function of concentration could be represented both by the linear equation, $\rho = 1.009118 + 0.002672c$, with an average deviation of 0.000012 g/cm³ and by the quadratic expression $\rho = 1.009115 + 0.002788c - 0.000227c^2$ with an average deviation of 0.000009 g/cm³. In both expressions, the protein concentration c is given in g/dl. Even though the quadratic expression did give a slightly better fit, the difference between the average deviations of these two representations is less than the estimated accuracy of the measurement. Because the linear expression was nearly as good as the quadratic one and because $(\partial\rho/\partial C)_\mu$ would have been concentration dependent had the quadratic expression been used, the linear representation was chosen. The calculated value for ϕ' ($\doteq \bar{v}$) was 0.726 ± 0.003 cm³/g.

Ultracentrifugation. All ultracentrifuge experiments were performed with a Spinco Model E analytical ultracentrifuge. The mechanical gear box speed control unit maintained the rotor speeds (calculated from the odometer readings) to within 5 rpm of the average speed at a nominal setting of 16,200 rpm, and to within 0.7 rpm of the average speed at nominal settings of 9341 rpm and below. The speed settings for these experiments ranged from 16,200 rpm to 5227 rpm;

rotor speed fluctuations were minimized by use of the 22 lb An-J rotor. Temperatures were controlled at 25.0 ± 0.03° by the standard RTIC unit, whose calibration was checked before and immediately after the series of experiments had been performed. The discrepancy of 0.02° between the two calibrations was judged to be negligible.

The optical system, which was the standard Rayleigh–schlieren arrangement, was aligned and focused on the midplane of the 12-mm cell according to a standard procedure (Gropper, 1964). Calculations indicate that focus of the camera lens on the midplane caused no significant perturbation of the data arising from Wiener skewness. The condensing lens mask was equipped with a symmetrically placed double slit. The cell thickness was calculated by measuring the thickness of the centerpiece with a comparator, and then diminishing this number by 0.5% to allow for cell compression (Nichol, 1968). Sapphire cell windows were employed for all experiments. Fringe displacements measured from blank experiments at each speed were consistently used to correct for window distortion. The wavelength of the light produced by the AH-6 lamp and the Wratten 77A filter arrangement was taken to be 550 mμ (Gropper, 1964). All images were registered on Kodak Type II-G spectroscopic plates.

Experiments were performed with 12-mm aluminum-filled epon centerpieces, except in two cases for which concentrations were less than 0.1 g/dl. For these, a 30-mm centerpiece was employed. No layering fluid was used in any of the experiments. The sectors were loaded with a syringe microburet after first rinsing the solution sector with protein solution in the manner recommended by Adams (1967). Use of polyethylene needles reduced the contact of the protein solutions with metal. The solvent sector was filled with a portion of the final dialysate. In all cases, the column heights in the solution sector were close to 0.3 cm. The calculated time to equilibrium was 16 hr, but the experiments were allowed to proceed for at least 26 hr and usually for 33 hr before the final photograph was made. The Rayleigh fringe patterns were checked for attainment of equilibrium, and the column heights from a final schlieren photograph were compared with those of an initial photograph in order to detect any leakage.

Results

From experiments at fifteen different initial concentrations, the quantities

$$M_{w(c)}^{app} = \frac{2RT}{(1 - \bar{v}\rho_0)\omega^2} \frac{d \ln c(r)}{d(r^2)} \qquad (13)$$

were calculated from slopes of the ln $c(r)$ *vs.* r^2 plots by use of the procedure described by Hancock and Williams (1969).[1] These plots were so nearly linear that frequently the curvature was almost obscured by plate-reading errors. Nevertheless, Figure 1 shows that the determinations of $M_{w(c)}^{app}$ as a function of c from experiments at different initial concentrations are consistent. Not shown are the results of three additional experiments, which were judged to be in error. Since the data from the 15 experiments match satisfactorily to form the locus of a continuous curve within the expected precision of the experiment, it may be concluded that the calculation procedure is adequate, that the sample is sufficiently pure to warrant analysis, and that the chemical equilibrium is rapidly reversible.

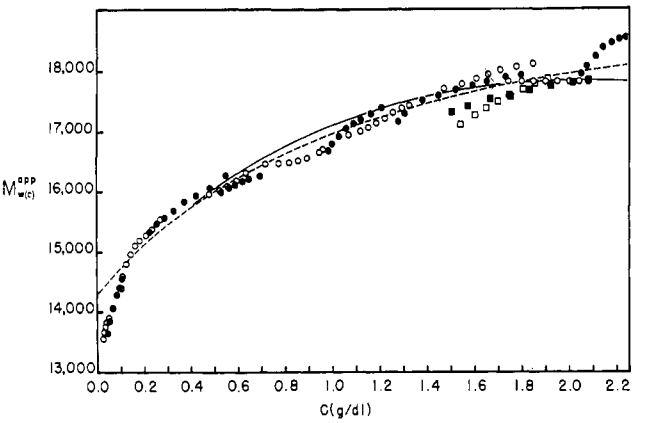

FIGURE 1: The apparent weight-average molecular weight of muramidase in NaCl–cacodylate buffer at pH 7.0, I = 0.20, and 25° as a function of concentration. Each set of symbols applies to a different experiment, with different symbols being employed to distinguish the experiments from each other. The width of the symbols is half the estimated experimental precision. The theoretical curve for a monomer–dimer association scheme (K_2 = 0.347 dl/g, BM_1 = 0.02 dl/g) is shown as a broken line, and the one for an indefinite association (k = 0.200, BM_1 = 0.09 dl/g) is shown as the solid line. Up to 0.4 g/dl, the values for $M_{w(c)}^{app}$ are practically identical for the two mechanisms.

The values of $M_{w(c)}^{app}$ for numerical analysis were obtained from an interpolation curve, which was drawn such that it was continuous and passed through the average ordinate at those concentrations for which there were more than two experiments. No attempt was made to ensure that derivatives of the interpolation curve with respect to c would be continuous. The data below 0.1 g/dl, which were obtained by use of a 30-mm cell are considered unreliable, and the interpolation curve was drawn horizontally in this concentration range so that it intercepted the ordinate at 14,307 g/mole, the monomer weight determined by amino acid analysis (Canfield, 1963; Canfield and Liu, 1965). The values for $M_{w(c)}^{app}$ at intervals of 0.1 g/dl are given in Table I. The average deviation of the data from the interpolation curve was 130 g/mole.

Indefinite Self-Association. Apparent weight-average molecular weight data (Table I) at intervals of 0.2 g/dl were taken from the interpolation curve for an analysis based upon eq 3 and the method described by Van Holde and Rossetti (1967). The 11 points were fit to an appropriate linear equation by use of the least-squares method to ascertain the best value for BM_1. The best estimate of BM_1 lies between 0.090 dl/g and 0.091 dl/g; corresponding to the latter value for BM_1 one obtains 0.7995 dl/g for the average value of $4k$. In Figure 2, $4k$ is plotted as a function of c. Most of the data are taken from near the meniscus, the center, and the bottom of the cell for each of 13 experiments. It may be seen that for a linear representation of these data, BM_1 = 0.091 is adequate. Deviations

[1] Because of the way in which $\bar{v}$ was determined, the term $(1 - \bar{v}\rho_0)$ in eq 13 is equivalent to $(\partial\rho/\partial C)_\mu$ with C being the concentration in g/cm³, provided that the solution density may be expressed adequately in the form $\rho = \rho_0 + (1 - \bar{v}\rho_0)C$. The term $(1 - \bar{v}\rho_0)$ served to apply corrections for the density of the dialysate actually used in the ultracentrifuge experiments.

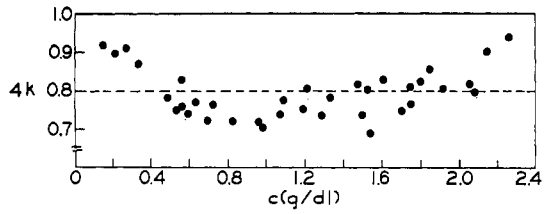

FIGURE 2: $4k$ (Indefinite mechanism) as a function of c, calculated by use of eq 3 and data indicated in text. The broken line corresponds to the value of $4k$ estimated from eleven points taken from the interpolation curve at equal concentration intervals.

from linearity are of the same order of magnitude as the scatter of the data. The value of $4k$ quoted above is somewhat larger than would be obtained from Figure 2 by averaging because the interpolation curve weights each concentration interval equally. The values for $M_{w(c)}^{app}$ predicted for an indefinite self-association, $BM_1 = 0.091$ dl/g, $4k = 0.7995$ dl/g, are plotted as the solid line in Figure 1. The average deviation of the apparent molecular weight data from the theoretical indefinite self-association curve is 200 g/mole, which is of the same order of magnitude as the experimental error.

Monomer–Dimer Association. Analysis of the apparent weight-average molecular weight data in terms of the monomer–dimer model is based upon eq 10. The functions F(c) and G(c) (which was obtained by quadrature of F(c) by use of Simpson's rule) are listed in Table I. It should be noted that G(c) is related to the apparent number-average molecular weight, $M_{n(c)}^{app}$, by the equation

$$G(c) = 2c(M_1/M_{n(c)}^{app}) - c \qquad (14)$$

The quantities U and V required by eq 10 are also given in Table I, along with the final values of W calculated in the last step of the iteration process. The quantities $2/a$, K_2, and BM_1 ($= b$) obtained from each cycle of the linear least-squares iteration procedure based upon eq 10 are presented in Table II. It is seen that for this case, the convergence is sufficiently rapid to make the procedure practical. Because of the term $1 - F(c)$ in the denominator of U, experimental error is magnified at low concentrations, but it was found practical to apply the procedure to the concentration range 0.2 g/dl to 2.2 g/dl. Thus only 13 experiments were actually used to determine the quantities K_2 and BM_1. The final values obtained for these parameters were inserted into eq 4 to generate the $M_{w(c)}^{app}$ *vs.* c curve predicted for the monomer–dimer model. This is displayed as the broken line of Figure 1. The average deviation of the experimental data from the theoretical monomer–dimer curve is 160 g/mole. Below 0.4 g/dl, the values of $M_{w(c)}^{app}$ calculated for the monomer–dimer mechanism and for the indefinite mechanism were nearly the same.

To allow an additional comparison of the monomer–dimer model to the indefinite self-association, $4K_2$ was calculated from eq 11, using $BM_1 = 0.0199$ and the same data that was employed in Figure 2. A plot of $4K_2$ as a function of c is shown in Figure 3. The values $K_2 = 0.347$ dl/g and $BM_1 = 0.0199$ dl/g from the previous analysis will differ slightly from those values obtained from eq 11 because the data are weighted differently for the two calculation methods.

General Monomer–n-mer Associations. For ideal solutions, it has been shown (Elias and Bareiss, 1967) that the aggregation number n of the n-mer for a monomer–n-mer reaction may be written as follows

$$n = \frac{(M_n/M_1)(M_w/M_1 - 1)}{(M_n/M_1 - 1)} \qquad (15)$$

where M_n and M_w are the true number-average and weight-average molecular weights, respectively, of the solute component. The corresponding apparent molecular weight functions from the experiments described above have been sub-

TABLE I: The Quantities Used for the Analysis of the Association of Muramidase.[a]

c (g/dl)	$M_{w(c)}^{app}$	F(c)	G(c)	U	V	W
0.10[b]	14,470	0.9775	0.0996	4.3271	1.3209	
0.20	15,260	0.8751	0.1916	1.3424	0.8938	−0.0909
0.30	15,610	0.8331	0.2767	1.3812	1.4441	−0.1020
0.40	15,840	0.8064	0.3586	1.4937	2.1481	−0.1171
0.50	16,030	0.7850	0.4382	1.5999	2.9507	−0.1317
0.60	16,190	0.7674	0.5158	1.7017	3.8489	−0.1458
0.70	16,330	0.7522	0.5918	1.7964	4.8310	−0.1594
0.80	16,440	0.7405	0.6664	1,9016	5.9350	−0.1736
0.90	16,580	0.7258	0.7398	1.9582	7.0005	−0.1844
1.00	16,790	0.7042	0.8114	1.9317	7.8668	−0.1894
1.10	17,080	0.6753	0.8802	1.8306	8.4803	−0.1893
1.20	17,240	0.6597	0.9468	1.8354	9.4691	−0.1964
1.30	17,380	0.6464	1.0121	1.8502	10.5313	−0.2040
1.40	17,480	0.6370	1.0763	1.8887	11.7414	−0.2132
1.50	17,570	0.6286	1.1396	1.9288	13.0135	−0.2224
1.60	17,580	0.6276	1.2028	2.0271	14.6500	−0.2355
1.70	17,710	0.6157	1.2649	2.0265	15.8211	−0.2413
1.80	17,830	0.6048	1.3259	2.0291	17.0365	−0.2472
1.90	17,940	0.5950	1.3859	2.0361	18.3072	−0.2533
2.00	18,050	0.5853	1.4449	2.0393	19.5823	−0.2589
2.10	18,150	0.5765	1.5030	2.0460	20.9091	−0.2646
2.20	18,240	0.5688	1.5602	2.0581	22.3049	−0.2706

[a] Additional figures have been retained to reduce rounding error. [b] This concentration was omitted from the calculation because the corresponding value of U was considered to be in error.

TABLE II: The Parameters Describing the Association of Muramidase According to the Monomer–Dimer Model for Several Iterations i. Calculation Based upon Equation 10.

i	$2/a$ (g/dl)	K_2 (dl/g)	$b = BM_1$ (dl/g)
1	1.5539	0.322	0.0277
2	1.3815	0.362	0.0196
3	1.4462	0.346	0.0203
4	1.4349	0.348	0.0198
5	1.4410	0.347	0.0199

TABLE III: The Apparent Aggregation Number n^{app} for Several Concentrations of Muramidase.

c (g/dl)	n^{app}
0.20	3.17
0.40	2.07
0.60	1.88
0.80	1.79
1.00	1.84
1.20	1.94
1.40	1.92
1.60	1.84
1.80	1.87
2.00	1.89
2.20	1.89

stituted into this equation to obtain an indication of which monomer–*n*-mer model provides the best description of the data. The apparent values for *n*, which are given in Table III for several concentrations, are seen to be close to 2 except at 0.2 g/dl, where experimental error becomes relatively more important.

Discussion

The data reported here display two improvements over previous efforts for muramidase which employ the ultracentrifuge: (1) the values for the apparent weight-average molecular weight plotted as a function of concentration overlap better to form a continuous curve, and (2) the molecular weight data for the reliable concentration range are in better accord with the monomer weight of muramidase based upon amino acid analysis. Of these, the first item is probably a result of the scrupulous preparative procedures: after three crystallizations, an additional chromatographic step was employed. [It should be mentioned that the discontinuities in the $M_1/M_{w(c)}^{app}$ *vs.* *c* plot shown by Adams and Filmer (1966) could arise from the presence of a higher molecular weight impurity which does not participate in the chemical reaction.] The second comment is based upon the use of the new value for the partial specific volume. By use of a lower value for this quantity, previous investigators (Sophianopoulos and Van Holde, 1964; Adams and Filmer, 1966) observed that their apparent molecular weight data under nonassociating conditions or extrapolated to zero concentration were over 1000 g/mole lower than the monomer weight to be expected on the basis of the amino acid analysis. The values for $\bar{v}$ at 25° reported in the literature range from 0.688 cm³/g (Colvin, 1952) to 0.723 cm³/g (Charlwood, 1957). With the application of the value reported here (0.726 cm³/g), which agrees with the larger of Charlwood's results within experimental error, the data from these ultracentrifuge experiments at concentrations greater than 0.2 g/dl are in good agreement with the monomer weight based upon chemical analysis.[2] These agreements, together with Mc-

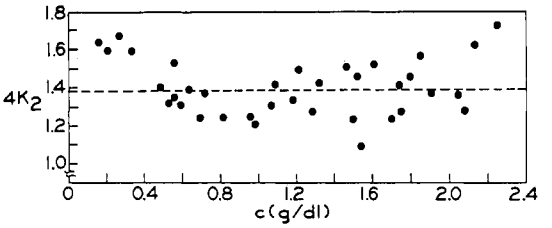

FIGURE 3: $4K_2$ (Monomer–dimer mechanism) as a function of *c*. Equation 11 and the same data used for Figure 2 were employed. The broken line represents the value of $4K_2$ obtained by use of eq 10. Note that the ordinate scale in this figure is one-half that of Figure 2.

Meekin's calculated value (0.718 cm³/g, 25°) (McMeekin *et al.*, 1964) suggest that the correct value for $\bar{v}$ for muramidase is indeed in the neighborhood of 0.72 cm³/g.

The equation of Elias and Bareiss indicates that of the possible monomer–*n*-mer representations, the monomer–dimer model provides the best description of our data. Any systematic variations of the aggregation number *n* with concentration would indicate either that the monomer–*n*-mer reaction is an inappropriate description, or that the system possesses considerable solution nonideality. It is seen that in Table III, the tendency is for *n* to be less than 2, which is taken to be an indication of solution nonideality. Because there are no other large systematic variations with concentration, these results indicate that the monomer–dimer model will provide a quite good representation of the experimental data.[3]

Even though the present data approach the expected practical limit of precision for the ultracentrifuge, it is evident from Figures 1, 2, and 3 that for this particular protein and buffer system, it is impossible to distinguish with certainty between the monomer–dimer reaction and an indefinite self-association reaction, given the scatter of the data and the concentration range covered. From Figure 1 it might be concluded that the monomer–dimer representation gives a somewhat better fit, but the difference between it and that of the indefinite self-association is not great. A comparison of Figures 2 and 3 also illustrates the difficulty in distinguishing between the two association schemes. The problems associated with the assignment of a mechanism to apparent molecular weight data will be less acute when the association constants are somewhat larger. With larger association constants, the range of apparent molecular weights observed over the concentration interval accessible to the Rayleigh optical system would be larger in comparison with experimental error than the range observed for muramidase under the present experimental conditions. Under such circumstances, any higher molecular weight species would be more abundant, and therefore it is expected that discrimination between alternative models would be facilitated. If the precision of our data could somehow have been improved or if the concentration range had been extended beyond 2.2 g/dl, then the distinction between these two possible mechanisms would also have been more clear-cut.

[2] Optical imperfections become relatively more important at low concentrations. Thus, data below 0.2 g/dl are subject to large errors, since only 2 to 4 fringes were observed with the 30-mm cell.

[3] We note in passing that an expression recently presented by Roark and Yphantis (1969), Teller *et al.* (1969), and Chun and Kim (1970), which may be used as the basis of a plot of $M_{w(c)}M_1$ *vs.* $M_1/M_{n(c)}$ to evaluate *n*, is a rearrangement of the equation for *n* presented by Elias and Bareiss (1967) which we have used here.

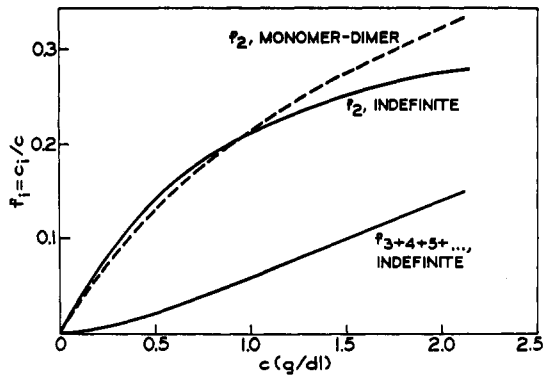

FIGURE 4: Weight fraction of dimer as a function of concentration for the monomer–dimer mechanism (broken line) and weight fractions of dimer and of all species larger than dimer for the indefinite mechanism (solid lines). The values for K_2 and k used in this calculation are the ones given in Figure 1.

We did not conduct experiments at higher concentrations, for several reasons: (1) the Rayleigh fringes were becoming blurred, (2) high concentration measurements might introduce complications from concentration dependence of $\bar{v}$ and of (dn/dc), and (3) it was unknown at just what point the third virial coefficient would make a significant contribution.

By using independent experimental techniques, it might be possible to resolve better the question of which mechanism actually operates for the muramidase self-association. Of course when the amount of association is small, it will be difficult to make a definitive statement of the mechanism regardless of the kind of data which are employed. Those measurements which are particularly sensitive to higher molecular weight species are most likely to provide the required information on which to base a selection. Van Holde and Rossetti have suggested that the investigation of a different molecular weight average would afford a semiindependent criterion for the assignment of the mechanism. From our data, we could have calculated both $M_{n(c)}^{app}$ and $M_{z(c)}^{app}$, but we did not do so because we have not yet fully accepted the notion that independent information is obtained from use of these additional molecular weight averages when data are imprecise and all derive from the same physical measurement. If we had obtained $M_{n(c)}^{app}$ by osmometry, an independent check would have been provided, but of course such data would be even less sensitive to the presence of species larger than dimer than is $M_{w(c)}^{app}$

Examination of the last figure presented by Van Holde *et al.* (1969) reveals that there are additional grounds for uncertainty about the assignment of a model to this system. There it is shown that in a case for which the indefinite mechanism provides an adequate description, the monomer–dimer–trimer and monomer–dimer–trimer–tetramer mechanisms also are satisfactory. This suggests that the present data might be described by some discrete model providing for species in addition to dimer at least as well as by the indefinite scheme. Provision for additional adjustable parameters no doubt would permit fits which are as good as the one for the monomer–dimer model. Put in another way, the monomer–dimer and the indefinite mechanisms might be thought of as extreme cases: since they both fit the data reasonably well, then intermediate mechanisms probably would fit also. We did not test any other possibilities because we had no means for judging these to be any more adequate than the two-parameter models already employed. An explanation of the data by assuming that species higher than dimer may be present was also given by Bruzzesi *et al.* for their light-scattering data for muramidase in phosphate buffer and up to concentrations of 4.5 g/dl (Bruzzesi *et al.*, 1965). Unfortunately, the precision of their data leaves something to be desired, and it seems possible that their data might be fit by using a monomer–dimer model with a somewhat larger value for K_2 than the one that they used.

The smallness of BM_1 obtained from the monomer–dimer analysis (0.02 dl/g) also raises some doubt regarding the appropriateness of that mechanism. If BM_1 were known independently for the muramidase monomer, then a comparison of this value with those obtained by analysis according to any mechanism would provide an additional criterion for the assignment of mechanism. In the present case, no independent value is available for our buffer conditions, so we resort to the less satisfactory procedure of comparison with values for BM_1 obtained experimentally for other globular proteins near zero charge and at high salt concentration. From a table compiled by Tanford (1961) it is seen that for four of the lower molecular weight proteins under these conditions, BM_1 ranges from 0.03 to 0.07 dl per g (taking into account the factor two associated with weight-average measurements). This range, which corresponds to the values expected from excluded volume contributions to solution nonideality, should indicate a lower bound for values of BM_1 obtained from mechanisms which adequately account for all association which is occurring. Since muramidase will likely be charged under the present experimental conditions (Tanford and Wagner, 1954), BM_1 is expected to exceed this range. The value of BM_1 obtained from analysis by means of the indefinite association scheme (0.09 dl/g) is in better accord with these expectations, which suggests that species higher than dimer may actually be present. Because we have very limited knowledge about BM_1 under such circumstances, however, such an interpretation must be accepted with reservation.

The indefinite self-association mechanism may be compared to the monomer–dimer mechanism by noting that for the indefinite case, the formation of dimer is described by the equation, $c_2 = 2kc_1^2$. Thus $2k$ is seen to play the role of a dimerization constant. By using k determined from equal weighting of concentration intervals and K_2 determined from eq 10, it is found that $2k = 0.400 \pm 0.031$ dl/g and $K_2 = 0.347 \pm 0.036$ dl/g. The precision indices are average deviations from the mean, calculated by use of eq 3 and 11 and data from three points in the cell for each experiment. It appears that in this case, estimation of the dimerization constant can be accomplished to a first approximation even if the two alternative models are different. Indeed, this has been observed previously by Hancock and Williams (1969), who found that for chymotrypsinogen A, K_2 for a monomer–dimer–trimer mechanism did not differ appreciably from $2k$ calculated from an indefinite association mechanism. Similarly, Van Holde *et al.* (1969) have found that K_2 for the indefinite (isodesmic), the monomer–dimer–trimer–tetramer, and the monomer–dimer–trimer mechanisms is roughly the same for their cytidine data regardless of which of these descriptions is selected.

Although it is not completely evident why two different mechanisms should give nearly the same values for the dimerization constant, this circumstance can be explained in part

by noting that sufficient conditions for having an equally good fit of experimental data at low concentrations from two different mathematical models are that both the initial slopes and the intercepts, respectively, of $M_{w(c)}^{app}$ predicted by these models be approximately the same. Considerations of the type given by Adams and Williams (1964) show that for the monomer–dimer case, the initial slope of a plot of R_a *vs.* c is $K_2 - BM_1$, while the initial slope for the indefinite association may readily be shown by series expansion to be $2k - BM_1$ (Adams, 1967). For the two cases described here, the values for BM_1 are small enough that having the initial slopes roughly the same requires that $2k$ and K_2 will be fairly close to each other. The indistinguishability at low concentrations is also illustrated by the standard plots presented by Chun and Kim (1970), from which it is seen that the curve for the indefinite association approaches closely the monomer–dimer curve as $M_{w(c)}/M_1$ approaches unity (*i.e.*, as c approaches zero).

The data of Figure 4 are also suggestive of the reason for the similarity in the two descriptions at low concentrations: under such conditions, the molecular weight distributions are nearly identical. In fact, the amounts of dimer predicted by the two mechanisms are approximately the same for both up to 2.0 g/dl. However, the increasing amounts of higher species predicted by the indefinite mechanism become relatively important even at moderate concentrations because they receive heavier weighting than the dimer in the weight-average measurements. This allowance for higher species is one reason why a larger value of BM_1 was required to fit the data to the indefinite mechanism than was required for the monomer–dimer mechanism. The requirement for larger values of BM_1 for the indefinite mechanism than for a discrete mechanism was also observed by Hancock and Williams (1969) for chymotrypsinogen A; however, the analysis of cytidine data (Van Holde *et al.*, 1969) indicates that this is not a general rule.

Although it has been possible on both mathematical and physical grounds to understand why $2k$ and K_2 should agree as well as they do when calculated from data at low concentrations, we have not yet gained a complete understanding of why they should agree when they have been estimated from data covering an extended concentration range. When it is understood what governs the magnitudes of the dimerization constants obtained from different mathematical models, it may be possible to proceed with thermodynamic calculations for the dimerization step without exact knowledge of which and how many of any higher species are present. Until this question has been fully answered, ambiguity with respect to mechanism discourages us from attempting any further analysis of our present data.

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