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THE INDEFINITE SELF-ASSOCIATION OF LYSOZYME: CONSIDERATION OF COMPOSITION-DEPENDENT ACTIVITY COEFFICIENTS

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An improved iterative method for computing association constants from sedimentation equilibrium results obtained with self-interacting protein systems is presented which accounts for the composition-dependence of the activity coefficients of all oligomeric species. The method is based on the calculation of virial coefficients from covolume and charge considerations, the statistical mechanical basis of which is discussed in relation to the DLVO theory. The method is applied to results obtained with lysozyme in diethylbarbiturate buffer of pH 8.0 and ionic strength 0.15 at 15°C. It is shown that these results, encompassing a range of total solute concentration up to 19.7 g/liter are consistent with self-association patterns comprising either a monomer–dimer–trimer system or an isodesmic indefinite self-association of the monomer, the latter being favored. A firmer distinction between these possibilities is sought on the basis of the dependence of the weight-average partition coefficient, determined by frontal gel chromatography, on total solute concentration (up to 56.6 g/liter). This analysis accounts for the composition-dependence of the ratio of the activity coefficients of partitioning monomer in mobile and stationary phases. It is concluded that all results are consistent with an indefinite self-association of lysozyme governed by a single association constant of 4.61×10^2 liter/mole.

1. Introduction

The elucidation of the self-association pattern of a protein requires experiments to be conducted over a relatively large range of total concentration since several models may appear to fit a limited set of results, especially those pertaining to low total concentrations [1–3]. Since experiments referring to relatively high total concentrations may be required as part of a set, their interpretation demands that attention be given to non-ideality effects [4–6]. One approach has been to express the logarithm of the activity coefficient of each oligomer as a power series in total concentration and to analyse sedimentation equilibrium results, for example, in terms of the oligomers present, the association equilibrium constants and the coefficient(s) of the power series suitably truncated [6–9]. This exercise in curve-fitting led to the interpretation of such results obtained with lysozyme at pH 6.7 in terms of a monomer-dimer system with an apparent second virial coefficient of negative sign [8, 10]. A similar interpretation [10] was given to results obtained at pH 8.0, closer to the isoelectric point of pH 11 [11]. It has been pointed out [12] that a negative second virial coefficient has no physical significance and that this difficulty could be overcome by interpreting the results in terms of a monomer–dimer–trimer system: the latter work [12], however, in so-doing, completely neglected non-ideality effects.

A second approach to the treatment of non-ideality is to express the logarithm of the species activity coefficients as functions of solution composition, which permits identification of the constant coefficients of the resultant power series as quantities which may be evaluated numerically on the basis of covolume and charge interaction considerations [13, 14]. It is true that such calculations require assumptions to be made concerning the geometry of oligomers and the net charges borne by them, but the advantage accrues that experimental results may now be analysed to yield solely the parameters governing the equilibria operative in solution, due allowance having been made

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for realistic estimates of the composition-dependent activity coefficients. Such a procedure for the analysis of sedimentation equilibrium results has been outlined theoretically [14] and applied [2] to the self-associating insulin system at pH 7.0. It is made tractable by the finding that the radial distribution of the thermodynamic activity of monomer can be evaluated directly from the sedimentation equilibrium distribution without assuming a model for the self-association [12]. The study on insulin [2], however, referred only to a limited range of total concentration and therefore considered only second virial coefficients: moreover, as the net charge borne by the insulin monomer was only −2, a relatively crude estimation of the contribution of charge interaction to the virial coefficients sufficed.

It is a major purpose of this work to elaborate on the calculation of virial coefficients and to illustrate the general importance of so-doing by reference to the self-association of lysozyme at pH 8.0 where the monomer of molecular weight 14400 [3] bears a net charge of +7.3 [11]. Sedimentation equilibrium results up to a total concentration of 19.7 g/liter are analysed for the first time with allowance for composition-dependent activity coefficients and the results are correlated with the concentration-dependence of the weight-average partition coefficient of the system determined by frontal gel chromatography. This correlation extends the examined concentration range to 56.6 g/liter.

2. Theory

Consider a solution containing monomer of molecular weight M_1 in equilibrium with a series of polymeric species. The total weight concentration, $\bar{c}$ (g/liter), is given by

$$\bar{c} = \sum_i c_i = M_1 \sum_i i \left(\prod_{l=1}^{l=i} K_l \right) a_1^i / y_i \,, \qquad (1)$$

where c_i is the weight-concentration of species i ($i = 1$, monomer; $i = 2$, dimer; etc.), a_1 is the thermodynamic activity of monomer referred to a standard state on the molar concentration scale and y_i is the activity coefficient of species i. In these terms, the successive equilibrium constants are also defined on the molar scale as

$$K_i = a_i/a_{i-1}a_1 = y_i m_i / y_{i-1} m_{i-1} y_1 m_1 \,, \qquad (2)$$

where $m_i = c_i/iM_1$ and $K_1 = 1$ by reason of nomenclature. It will be assumed that no volume changes occur on self-association and thus that the equilibrium constants are independent of pressure. Eq. (1) encompasses consideration of all self-association patterns [12], those of particular relevance to this work being [3,8,12], a monomer–dimer system ($i = 1, 2$), a monomer–dimer–trimer system ($i = 1, 2, 3$) and an isodesmic indefinite self-association ($i = 1, 2, \ldots, \infty$ with all $K_i = K_2$ for $i > 2$ so that $\{\Pi K_l\} = K_2^{(i-1)}$).

Eq. (1) applies at every radial distance, x, in the cell of a sedimentation equilibrium experiment and is particularly appropriate to the analysis of such results since the $\Omega(x)$ method [12] may be used to determine $a_1(x)$ as a function of $\bar{c}(x)$ from a series of experiments spanning a range of total concentration. However, its use in a least-squares fitting procedure to determine the products of appropriate equilibrium constants, and hence individual best-fit values of K_i, requires comment on the $y_i(x)$ which are functions of the composition of the mixture and hence of $\bar{c}(x)$. Explicitly,

$$\ln y_i(x) = \sum_j \alpha_{ij} m_j(x) + \sum_j \sum_k \alpha_{ijk} m_j(x) m_k(x) + \ldots \,, \qquad (3)$$

where each of the subscripts $i, j, k, \ldots$ is allowed to span the set of oligomeric species independently. In this work higher terms in eq. (3) are neglected and detailed comment on the means used to evaluate the sets of constant coefficients $\{\alpha_{ij}\}$ and $\{\alpha_{ijk}\}$ are given in the Appendix. It suffices here to note that the set $\{\alpha_{ij}\}$, corresponding to second virial coefficients, are calculated on the basis of excluded volumes of equivalent spheres together with a consideration of charge interactions which is based on the assumption of conservation of charge on polymerization and on an accurate description of the electric potential of interaction between species i and j according to the DLVO theory [15,16]. The treatment effects an improvement of the charge correction term, $Z_i Z_j / 2I$, conventionally added to the covolume contribution in the estimation of second virial coefficients [2,13,14,17]. The method used to calculate the set $\{\alpha_{ijk}\}$, corresponding to third virial coefficients, involves numerical integration of an equation also encompassing covolume and charge effects: previous calculations of third virial coefficients have not considered the latter effect [18].

It is clear that the use of eqs. (1) and (3) in the elucidation of any self-association pattern from $(\bar{c}(x), a_1(x))$ data must be based on an iterative procedure. We suggest that a suitable starting point is the approximation, $\ln y_i(x) \sim \alpha_{ii}\bar{c}(x)/M_i$; for then approximate values of $y_i(x)$ for each oligomeric species may be calculated as a function of $\bar{c}(x)$ using the calculated values of α_{ii}. For the monomer–dimer or isodesmic indefinite self-association patterns, eq. (1) may then be fitted using a non-linear regression procedure to experimental $(\bar{c}(x), a_1(x))$ points in terms of an apparent value of the single equilibrium constant, K_2. The second step utilizes this estimate of K_2, and the experimental $a_1(x)$ values, in eq. (2) to calculate first approximations of $a_2(x)$, and of the activities of higher polymers in the case of indefinite self-association. These values, divided by the first estimates of $y_i(x)$, are substituted into eq. (3) in place of the required molar concentrations to give improved estimates of $y_i(x)$. It has been shown [14] that division of the available $a_i(x)$ by these improved $y_i(x)$ values permits eq. (3) to be used in an iterative manner which rapidly converges on the best estimates of $y_i(x)$ appropriate to the apparent value of K_2. These values of $y_i(x)$ may now be used in fitting eq. (1) to the experimental results whence an improved estimate of K_2 emerges. This procedure, which considerably extends that described previously [14] based on a single estimate of K_2, may be repeated until values of K_2 and of $y_i(x)$ become invariant within reasonable tolerance. Moreover, the same procedure is directly relevant to the examination of other models, such a monomer–dimer–trimer system, involving more than one equilibrium constant. It is now possible to compare the relevance of different models examined in this way by calculating apparent values of $\bar{c}(x)$ using eq. (1) and testing them directly against corresponding experimental values.

In principle, it is possible to conduct an extensive series of sedimentation equilibrium experiments with different loading concentrations and/or angular velocities to examine by overlapping steps [12] a very wide range of total concentration. This, however, is a formidable task in view of the resolving power of available optical systems, as has been noted specifically in relation to sedimentation equilibrium studies on lysozyme [1]. We suggest, therefore, that self-association patterns defined with precision from the analysis of a more limited set of sedimentation equilibrium results may be distinguished on the basis of results obtained at higher total concentrations by other experimental procedures. In particular, such a correlation is possible using weight-average partition coefficients determined as a function of total concentration by frontal chromatography in which a plateau of original concentration is maintained throughout the experiment [19,20]. It has been shown [21] that, provided a stationary matrix may be found which is not subject to noticeable osmotic shrinkage at the plateau values of total concentration, $\bar{c}^{\gamma}$, and which excludes all oligomeric species except monomer,

$$\sigma_w = \sigma_1 c_1^{\gamma}/\bar{c}^{\gamma} , \tag{4}$$

where σ_w is the weight-average partition coefficient determined from the median bisector of the advancing front. The partition coefficient of monomer is denoted by σ_1 which equals c_1^{β}/c_1^{γ} where c_1 is the weight concentration of monomer and the superscripts β and γ refer to the stationary and mobile phases, respectively. Eqs. (2a), (2b) and (4) of ref. [21] may be rearranged to show that σ_1 is related to σ_1^0, the partition coefficient of monomer at infinite dilution, by

$$\sigma_1 = \sigma_1^0 y_1^{\gamma}/y_1^{\beta} \exp(-G) , \tag{5}$$

where $\exp(G)$ is the contribution to the monomer activity coefficient in the stationary phase due to monomer-matrix interaction. In these terms, eq. (3) becomes

$$y_1^{\beta} = \exp(\alpha_{11} m_1^{\beta} + \alpha_{111}(m_1^{\beta})^2 + \ldots) \exp(G) . \tag{6}$$

Combination of eqs. (5) and (6) together with the relation $m_1^{\beta} = \sigma_1 c_1^{\gamma}/M_1$ yields,

$$\sigma_1 = \sigma_1^0 y_1^{\gamma}/\exp\{\alpha_{11}(\sigma_1 c_1^{\gamma}/M_1) + \alpha_{111}(\sigma_1 c_1^{\gamma}/M_1)^2 + \ldots\} . \tag{7}$$

The procedure for testing a self-association pattern deduced from sedimentation equilibrium analysis in relation to $(\bar{c}^{\gamma}, \sigma_w)$ results is as follows. For a series of values of $\bar{c}^{\gamma}$, the postulated equilibrium constant(s) relevant to the indicated self-association pattern may be used with eqs. (1) and (3) to obtain corresponding values of c_1^{γ} and y_1^{γ} by the iterative procedure previously described [14]. Thus, eq. (7) may be solved numerically for σ_1 values corresponding to selected $\bar{c}^{\gamma}$ values utilizing the value of σ_1^0 obtained by extrapolating σ_w values to infinite dilution. The relevance of the particular self-association pattern may now be

tested by calculating σ_w versus $\bar{c}^\gamma$ from eq. (4) and comparing the result directly with the experimental points.

3. Experimental

3.1. Lysozyme solutions

Salt-free lysozyme (hens' egg white) was obtained from Worthington Biochemical Corp. and used without further purification. All experiments were conducted with solutions of the protein in 0.15 I diethylbarbiturate buffer (0.02 M sodium diethylbarbiturate, 0.01 M diethylbarbiturate acid, 0.13 M sodium chloride), pH 8.0. Solutions were dialysed against this buffer using pre-washed Visking 8/32 cellophane tubing prior to use and concentrations were determined either spectrophotometrically employing an extinction coefficient ($E^{1\%}_{1cm}$) at 280 nm of 26.35 [22] or refractometrically using a synthetic boundary ultracentrifuge cell as described previously [10]. In the latter connection, the relationship $\bar{c} = 0.254$ J was employed [12] where $\bar{c}$ is in g/liter and J in Rayleigh interference fringes in a 12 mm path length cell: the assumption was made that the specific refractive increments of all oligomeric species of lysozyme were identical. Densities of lysozyme solutions were determined using an Anton-Paar DMA 02C precision density meter and proved to be linear with total concentration.

3.2. Sedimentation equilibrium

Experiments were performed using a Spinco model E ultracentrifuge fitted with electronic speed control and the temperature was maintained at (15 ± 0.1)°C with the RTIC unit and refrigerator. Rayleigh interferograms recording equilibrium distributions were measured with a two-dimensional Nikon microcomparator to obtain plots of $\bar{c}(x)$ versus x, as described earlier [10,23]. Three of the experiments had been performed previously [10] and the fourth was conducted using the same protocol, including the use of a solution column height of approximately 3 mm. These experiments are designated in terms of initial loading concentration (g/liter) and angular velocities (r.p.m.) as follows: expt. 1, 2.896 g/liter, 20 000 r.p.m.; expt. 2, 7.473 g/liter, 15 000 r.p.m.; expt. 3, 11427 g/liter, 11000 r.p.m. and expt. 4, 17, 142 g/liter, 8000 r.p.m.

The analysis of $\bar{c}(x)$ versus x plots obtained from these experiments differed from that described in the earlier work [10] in that the $\Omega(x)$ method [12] was employed to obtain the relationship between $a_1(x)$ and $\bar{c}(x)$ directly without estimating apparent weight-average molecular weights. In detail, a reference concentration $\bar{c}(x_F) = 4.826$ g/liter common to expt. 1 and 2 was selected which arose at $x_F = 7.1448$ cm and 6.9245 cm, respectively, to evaluate $\Omega(x)$ values according to eq. (5) of ref. [12]. The partial specific volume of lysozyme was taken as 0.726 ml/g [3,10,12]. A plot of $\Omega(x)$ versus $\bar{c}(x)$, which proved to be coincident in the range of $\bar{c}(x)$ common to both experiments, led to an extrapolated value of $\Omega^0(x)$ of 0.820 which permitted evaluation of $a_1(x)$ as $\bar{c}(x)\Omega^0(x)/\Omega(x)$. The plot of $a_1(x)$ versus $\bar{c}(x)$, thus obtained from expt. 1 and 2, extended up to a value of $\bar{c}(x)$ of 10.5 g/liter. Expt. 3 encompassed the $\bar{c}(x)$ range of 8.6–14.2 g/liter, part of which is evidently common to that in expt. 2. It thus follows that $a_1(x)$ was known for specified x values in expt. 3 within this common range, and accordingly, $a_1(x)$ versus $\bar{c}(x)$ was computed from eq. (3) of ref. [12] for the entire $\bar{c}(x)$ range encountered in expt. 3. Similarly, values of $a_1(x)$ from expt. 4, which encompassed the $\bar{c}(x)$ of 15.0–19.7 g/liter, were calculated by estimating the value of $a_1(x)$ at the meniscus position of expt. 4 by a small extrapolation of the $a_1(x)$ versus $\bar{c}(x)$ obtained from the preceding three experiments.

3.3. Exclusion chromatography

Dialysed solutions of lysozyme of known concentration, $\bar{c}^\gamma$, were subjected to frontal exclusion chromatography on a column (0.9 × 69 cm) of Sephadex G-50 (Pharmacia) pre-equilibrated with degassed diethylbarbiturate buffer of pH 8.0 and thermostatically maintained at 15°C. Approximately 30 ml of each protein solution (degassed and subjected to filtration using a 0.45 μ Millipore membrane) was applied to the column by upward flow, the flow-rate being maintained at 23 ml/h by means of a peristaltic pump. Fractions (1.0–1.2 ml) were collected in pre-weighed tubes so that the precise weight of each fraction could be determined and concentrations of lysozyme

in each fraction were determined spectrophotometrically. This permitted construction of elution profiles on a volume basis by use of the calibration curve of density versus concentration. The median bisector of the advancing front in each experiment was determined to give the weight-average elution volume, V_w, corresponding to the particular plateau concentration, $\bar{c}^\gamma$ [19]. The corresponding weight-average partition coefficient, $(\sigma_w)_{obs}$, was found as $(V_w - V_0)/(V_t - V_0)$, where V_0 was the void volume of the column (17.35 ml, determined with blue dextran) and V_t was the total accessible volume (45.25 ml, determined with K_2CrO_4).

Two further points merit comment in relation to these experiments. First, Sephadex G-50 was chosen as the column matrix since its exclusion limit of molecular weight ~30000 for globular proteins ensured that dimers and higher polymers of lysozyme were effectively excluded from the stationary phase, and because this highly cross-linked dextran is subject to little or no osmotic shrinkage [24]. Indeed, in the latter connection, it was observed that the total volume of the column remained invariant even in the presence of the highest concentration of lysozyme employed (56.6 g/liter). Secondly, it is known that in addition to a partitioning effect of lysozyme monomer on Sephadex columns, the protein also reversibly adsorbs to the gel matrix [25,26]. A correction for this effect was made as follows. The partition coefficients of a series of noninteracting globular proteins of molecular weight close to that of the lysozyme monomer, cytochrome c (11700), ribonuclease (13700), α-lactalbumin (14200) and myoglobin (17200), were determined separately in the same diethylbarbitutate buffer at low protein concentration (~0.2 g/liter). The values obtained were 0.30, 0.30, 0.26 and 0.24, respectively. On this basis, a value of 0.27 was chosen for σ_1^0, the partition coefficient of lysozyme monomer at infinite dilution corrected for the absorption effect. The corresponding observed value, which reflects both partitioning and absorption, was 0.50. The difference, 0.23, was subtracted from each $(\sigma_w)_{obs}$ obtained as a function of $\bar{c}^\gamma$ to yield the plot of σ_w versus $\bar{c}^\gamma$ reported later, where σ_w refers to a weight-average partitioning effect alone. In relation to this subtractive procedure, it is noted that the correction term employed closely resembles that of 0.25 found in previous studies [26] and that the method is seen from eq. (4) of ref. [26] to be justified in cases where the concentration of the unbound acceptor sites on the matrix is relatively insensitive to the applied concentration of protein.

4. Results

Fig. 1 presents the plot of the thermodynamic activity of lysozyme monomer, $a_1(x)$, with reference to a standard state on the molar scale, as a function of the total lysozyme concentration, $\bar{c}(x)$, obtained from sedimentation equilibrium expts. 1–4. The assessed accuracy of the points is ±0.04 g/liter, within the area of the symbols shown. It is evident that the results from different experiments are coincident in their common ranges of $\bar{c}(x)$, which directly implies that any volume changes on self-association are undetectable within experimental precision [12]. This finding justifies the assumption made in the theoretical section that association constants for the lysozyme system are independent of pressure, at least in the range 1–8 atm encompassed in the sedimentation equilibrium experiments, but seems to be at variance with the interpretation suggested earlier [10] that the self-association of lysozyme at pH 8.0 (but not at pH 6.7) is accompanied by an apparent negative volume change. The latter interpretation was based on small differences (≤3.5%) in apparent weight-average molecular weight results obtained at the same values of $\bar{c}(x)$ in different experiments and, as the authors noted [10], errors in the differentiation procedure used to obtain these values may well be an alternative cause for the observed non-superposition: this source of error has been obviated in the present analysis by the use of the $\Omega(x)$ method which is more direct and accurate [12].

The results shown in fig. 1 were analysed by the iterative procedure outlined above and based on eqs. (1) and (3), in terms of three self-association patterns, monomer–dimer, monomer–dimer–trimer and an indefinite self-association. These analyses required the evaluation of the sets of constant coefficients $\{\alpha_{ij}\}$ and $\{\alpha_{ijk}\}$, contained in eq. (3), utilizing relations given in the Appendix. Table 1 summarizes values of α_{ij} so obtained using values of the radii of equivalent spheres for the oligomeric species calculated from $r_i = (3iV_1/4\pi)^{1/3}$ where V_1 (2.0×10^{-20} cm^3) is the best esti-

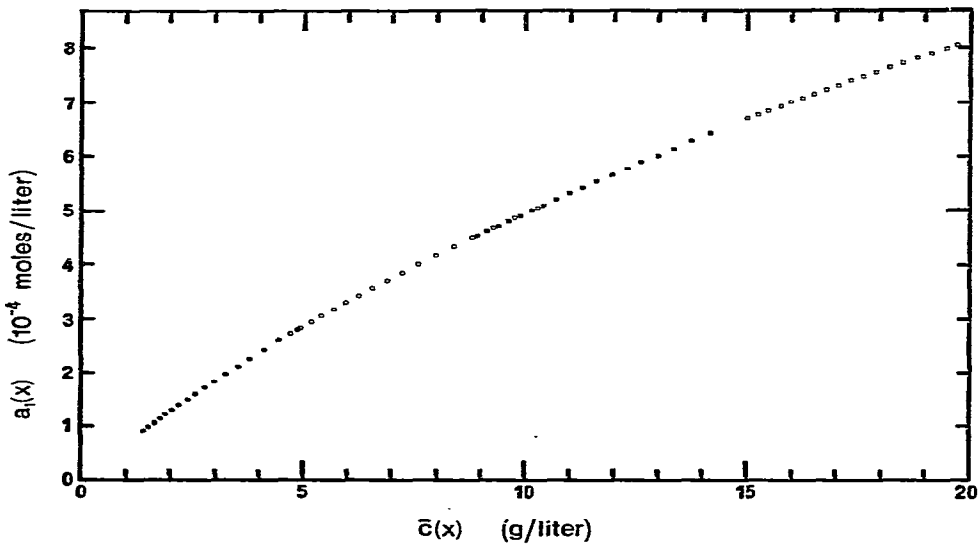

Fig. 1. The dependence of the thermodynamic activity of monomer, referred to a standard state on the molar scale, $a_1(x)$, on total weight concentration, $\bar{c}(x)$, obtained from sedimentation equilibrium experiments conducted with lysozyme at 15°C in diethylbarbiturate buffer, 0.15 I, pH 8.0. The experiments are delineated in the text and the use of symbols is as follows: • (expt. 1); ○ (expt. 2); ▪ (expt. 3); □ (expt. 4).

mate of the effective volume of a molecule of the lysozyme monomer [27]: in these calculations, Z_i was taken as $7.3i$. A discussion of one value from table 1 suffices to illustrate the relative magnitudes of terms contributing to it, as detailed in the Appendix, and we choose the monomer–monomer ($i = j = 1$) value of 2.1×10^2 liter/mole. This equals according to eq. (A.2) the difference between $2A_{11}$ (2.2×10^2 liter/mole) and $M_1\bar{v}$ (0.1×10^2 liter/mole), illustrating the general observation that the molar volume term is relatively small. In turn, $2A_{11}$ is comprised of the covolume effect of magnitude 1.0×10^2 liter/mole and the charge interaction term of 1.2×10^2 liter/mole, which stresses the importance of assessing the latter term re-

Table 1
Calculated values of α_{ij} corresponding to second virial coefficients for lysozyme as defined in eq. (3). The units are 10^2 liter/mole

	j									
i	1	2	3	4	5	6	7	8	9	10
1	2.1	3.3	4.3	5.1	5.9	6.6	7.3	7.9	8.5	9.1
2	3.4	5.1	6.5	7.6	8.7	9.7	10.5	11.4	12.2	12.9
3	4.5	6.6	8.2	9.6	10.8	12.0	13.0	14.0	14.9	15.8
4	5.4	7.9	9.7	11.3	12.7	14.0	15.1	16.2	17.3	18.2
5	6.3	9.0	11.0	12.8	14.3	15.7	17.0	18.2	19.3	20.4
6	7.2	10.1	12.3	14.2	15.8	17.3	18.7	19.9	21.2	22.3
7	7.9	11.1	13.4	15.4	17.2	18.8	20.2	21.6	22.9	24.1
8	8.7	12.0	14.5	16.6	18.5	20.2	21.7	23.1	24.5	25.7
9	9.4	12.9	15.6	17.8	19.7	21.5	23.1	24.6	26.0	27.3
10	10.1	13.8	16.6	18.9	20.9	22.7	24.4	25.9	27.4	28.8

alistically. In this connection, it is noted that this improved estimate of the charge interaction term obtained by numerical integration differs significantly from the value 1.8×10^2 liter/mole calculated from the conventional first estimate of the charge term, $Z_1^2/2I$ [2,13,14,17]. Values of α_{ijk} (one thousand in number) were computed from eqs. (A.3) and (A.10) in a range encompassing the decamer of lysozyme (as in table 1) and table 2 presents a subset of these values. For brevity, comment is again made only on a single value, $\alpha_{111} = 7.9 \times 10^3$ liter^2/mole^2, corresponding to the third virial coefficient for monomer–monomer–monomer interaction. This equals according to eq. (A.3) the difference between $3A_{111}/2$ (9.1×10^3 liter^2/mole^2) and $M_1\bar{v}A_{11}$ (1.2×10^3 liter^2/mole^2), showing again that the former term dominates. Of greater interest is the observation that α_{111} would have the value 1.7×10^3 liter^2/mole^2 in the absence of charge interactions: such values based on covolume considerations alone have been used [18] in relation to isoelectric protein studies, but the present comparison emphasizes the necessity of considering charge effects in the present context.

Best fit estimates of the association constants for the three models examined by the iterative analysis of the results in fig. 1 with the use of calculated α_{ij} and α_{ijk} values were as follows: monomer–dimer, $K_2 = (7.14 \pm 0.04) \times 10^2$ liter/mole; monomer–dimer–trimer, $K_2 = (3.58 \pm 0.04) \times 10^2$ liter/mole and $K_3 = (1.04 \pm 0.04) \times 10^3$ liter/mole; isodesmic indefinite self-association, $K_2 = (4.61 \pm 0.03) \times 10^2$ liter/mole. Fig. 2 presents plots of the best fit activity coefficients relevant to the models, those for the indefinite self-association pattern encompassing oligomers up to the decamer, since the contribution of higher polymers to the total concentration was shown to be negligible at $\bar{c}(x)$ values of 20 g/liter. Table 3 examines the relevance of these patterns in terms of comparisons of experimental values of $\bar{c}(x)$ and those calculated using eq. (1), the derived equilibrium constants, values of $a_1(x)$ from fig. 1 and the values of the activity coefficients (fig. 2). It is apparent from table 3, which pre-

Table 2
A subset of calculated values of α_{ijk} corresponding to third virial coefficients for lysozyme as defined in eq. (3). The units are 10^3 liter^2/mole^2

		k		
	j	1	2	3
i = 1	1	7.9	16.0	22.7
	2	15.0	28.1	42.1
	3	21.5	39.7	55.6
i = 2	1	15.3	29.9	42.1
	2	28.5	52.2	76.4
	3	40.3	72.9	101
i = 3	1	22.1	42.4	59.2
	2	40.6	73.5	106
	3	57.0	102	140

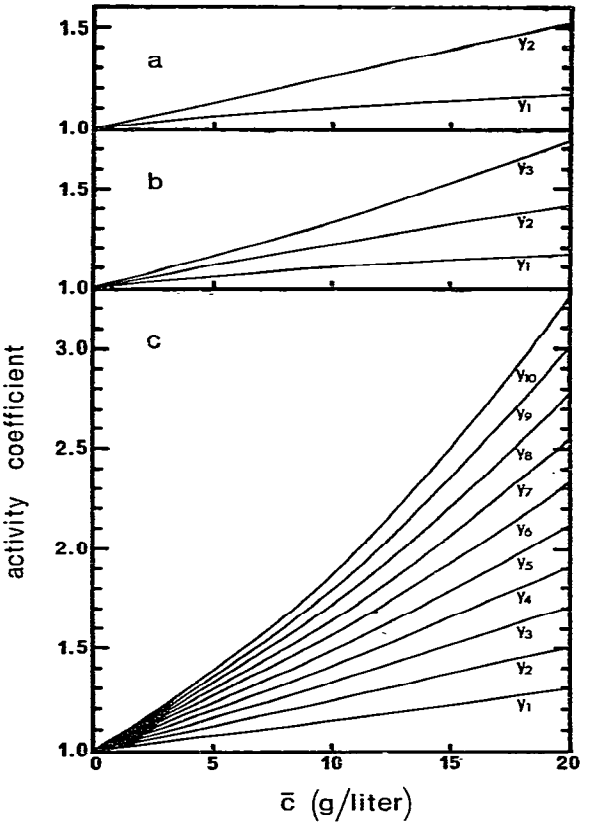

Fig. 2. The dependence of calculated activity coefficients of oligomeric species of lysozyme on total weight concentration. (a) monomer–dimer model with $K_2 = 7.14 \times 10^2$ liter/mole; (b) monomer–dimer–trimer model with $K_2 = 3.58 \times 10^2$ liter/mole and $K_3 = 1.04 \times 10^3$ liter/mole; (c) isodesmic indefinite self-association pattern with $K_2 = 4.61 \times 10^2$ liter/mole and curves referring progressively to oligomeric species from monomer to decamer as indicated.

Table 3
An examination of sedimentation equilibrium results obtained with lysozyme at pH 8.0 in terms of three possible self-association patterns a)

Experimental		Calculated					
$a_1(x)$ (10^{-4} moles/liter)	$\bar{c}(x)$ (g/liter)	monomer–dimer $\bar{c}(x)$	$\Delta\bar{c}$	monomer–dimer–trimer $\bar{c}(x)$	$\Delta\bar{c}$	indefinite $\bar{c}(x)$	$\Delta\bar{c}$
1.279	1.994	2.115	0.121	1.987	−0.007	2.016	0.022
2.374	4.013	4.311	0.298	3.981	−0.032	4.036	0.023
3.338	6.048	6.484	0.436	6.011	−0.037	6.062	0.014
4.151	7.988	8.452	0.464	7.933	−0.055	7.959	−0.029
4.927	9.975	10.415	0.440	9.946	−0.029	9.935	−0.040
5.694	12.045	12.417	0.372	12.097	0.052	12.053	0.008
6.365	13.995	14.204	0.209	14.100	0.105	14.044	0.049
7.013	16.053	15.947	−0.106	16.126	0.073	16.085	0.032
7.572	17.996	17.458	−0.538	17.936	−0.060	17.941	−0.055
8.033	19.700	18.705	−0.995	19.466	−0.234	19.537	−0.163

a) The patterns examined were as follows: monomer–dimer with $K_2 = 7.14 \times 10^2$ liter/mole; monomer–dimer–trimer with $K_2 = 3.58 \times 10^2$ liter/mole and $K_3 = 1.04 \times 10^3$ liter/mole; isodesmic indefinite self-association with $K_2 = 4.61 \times 10^2$ liter/mole.

sents a selection of results over the range of points in fig. 1, that the monomer -dimer model, previously proposed [10], is unacceptable and, indeed, a comparison of $\bar{c}(x)$ values based on the use of two hundred experimental ($\bar{c}(x)$, $a_1(x)$) points led to a standard deviation of ±0.392 g/liter for this model. On the other hand, the standard deviation, the function minimised in the iterative fitting, so calculated for the monomer–dimer–trimer and isodesmic systems was ±0.063 g/liter and ±0.039 g/liter, respectively. In this connection it is noted that a standard deviation of ±0.04 g/liter has been assessed [2] as reasonable in reflecting errors in the $\Omega(x)$ analysis of sedimentation equilibrium results. It follows that the pattern of isodesmic indefinite association governed by $K_2 = (4.61 \pm 0.03) \times 10^2$ liter/mole is favored by the present results. Clearly, however, while the monomer–dimer model need be considered no further, it would be advantageous to search for a firmer distinction between the monomer–dimer–trimer model, implying the formation of a cyclic trimer in termination of a definite self-association, and the model of indefinite self-association. This must be based on results obtained at even higher total concentrations than 20 g/liter.

Fig. 3 presents a plot of the weight-average partition coefficient of lysozyme, σ_w (corrected for adsorption effects as described in the experimental section) versus

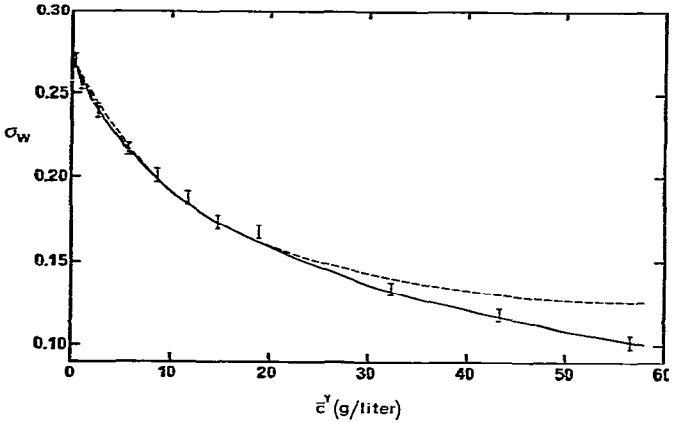

Fig. 3. The dependence of the weight-average partition coefficient of lysozyme, σ_w (corrected for absorption effects as described in the text) on total weight concentration in the plateau region. Experimental points were determined by frontal chromatography on Sephadex G-50 at 15°C in diethylbarbiturate buffer, 0.15 *I*, pH 8.0. The solid and broken curves refer, respectively, to the isodesmic indefinite self-association and monomer–dimer–trimer patterns and were calculated using the association constants reported in the caption to fig. 2 with allowance for composition-dependent non-ideality as described by eqs. (3) and (7).

the total plateau concentration, $\bar{c}^{\gamma}$, used in the frontal chromatrography experiments. The solid and broken curves in fig. 3 were computed for the isodesmic indefinite self-association pattern and the monomer–dimer–trimer system, respectively. In these calculations, values of the association equilibrium constants were those found from the analysis of the sedimentation equilibrium results and eqs. (4) and (7) were employed as previously described with $\sigma_1^0 = 0.27$, $\alpha_{11} = 2.1 \times 10^2$ liter/mole (table 1) and $\alpha_{111} = 7.9 \times 10^3$ liter^2/mole^2 (table 2). Calculation of y_1^{γ} and c_1^{γ} for use in eq. (7) required, for the isodesmic system at $\bar{c}^{\gamma}$ greater than 20 g/liter, consideration of oligomeric species larger than the decamer species since polymers up to the 15-mer were computed to contribute significantly to the highest $\bar{c}^{\gamma}$ of 56.6 g/liter. It is evident from fig. 3 that both self-association patterns examined fit the results within experimental error up to 20 g/liter and this is not unexpected in view of the dependence of composition on total concentration reflected by both models in the sedimentation equilibrium results in this concentration range. The fit in this region is reassuring in relation to the subtractive correction procedure used to obtain σ_w values. Above 20 g/liter, the theoretical curves in fig. 3 diverge and only that referring to the isodesmic indefinite self-association patterns passes through the experimental points.

5. Discussion

Several workers [3,8,10,12,28–31] have investigated the self-association of lysozyme in the pH range 6.7 to 8.0 where the net charge borne by the monomer varies by only one unit from +8.3 to +7.3 [11]. A range of values between 2×10^2 and 8×10^2 liter/mole for the dimerization constant was reported, the values decreasing with increasing temperature (4–25°C) and their magnitudes being sensitive to the various assessments of self-association patterns and of non-ideality effects. This range of values for K_2 corresponds to a standard free energy change for dimer formation of −3.0 to −3.8 kcal/mole. In general, it has been agreed that at least one polymer larger than the dimer coexisted in the equilibrium mixtures and Deonier and Williams [3] have referred to the possibility that an indefinite self-association was operative. In the latter connection, it could be noted that two studies [30,32] on the interaction of the competitive inhibitor, N-acetylglucosamine, with the lysozyme system have both been interpreted on the basis of a "head to tail" self-association of lysozyme involving one active site in the formation of dimer. Such a proposal which has been supported by nuclear magnetic resonance studies [33], implies that there is no a priori reason, except for net electrostatic repulsion, why a series of higher polymers of lysozyme may not be formed. In this work a test of this proposal was made by selecting conditions (a relatively high pH value and low temperature in the ranges previously investigated, a relatively high ionic strength of 0.15 and a large range of total concentration up to 56 g/liter) that would favor any high polymer formation. Moreover, particular attention has been paid to the treatment of non-ideality effects on the basis of composition-dependent activity coefficients expressed in terms of virial coefficients which reflect both covolume and charge contributions. The results obtained from sedimentation equilibrium (table 3) and from partition chromatography corrected for adsorption effects (fig. 3) both favor the operation of an isodesmic indefinite self-association of lysozyme governed by a single association constant of 4.61×10^2 liter/mole, corresponding to a standard free energy change of −3.5 kcal/mole for addition of monomer to form higher polymers. The possibility, however, cannot be excluded that the successive equilibrium constants (on a molar scale) may differ slightly in magnitude; for any such effect would evidently be within experimental error.

The point of more general significance in this work is the demonstration that composition-dependent non-ideality effects may be considered by improved iterative procedures for self-associating systems both in sedimentation equilibrium and in partition chromatography. In the latter case, previous treatments of non-ideality have been based on one of the following assumptions: (1) that the weight-fraction of monomer is large in the range of total concentration encompassed [34], so that virial coefficients calculated from covolume considerations for the partitioning monomer sufficed to express the activity coefficient of the monomer as a function of total concentration; or (2) that the Adams–Fujita approximation [6], $\ln y_i = iBM_1\bar{c}$, where B is an apparent virial coefficient, was valid in a range of relatively low total concentration [21]. The reasonableness of the latter approximation may be

judged on the basis that the ratio $y_i/y_{i-1}y_1$, approximates unity [6,14], a condition which may be assessed from fig. 2 to be of decreasing validity as large polymers or high total concentrations are considered. Moreover, use of this approximation written for a two-term virial expansion in fitting the sedimentation equilibrium results of the present study led to at least one negative value of the apparent virial coefficients for each model examined. With the requirement removed that these coefficients be positive on a physical basis, it followed that it was no longer possible to decide between the models on the basis of the experimental results. This highlights the necessity of providing a realistic physica basis for the estimation of the virial coefficients which delineate the composition-dependence of the activity coefficients. It is hoped, therefore, that the theory presented in the Appendix, its numerical illustration in tables 1 and 2, and its iterative use in relation to eqs. (1), (3) and (7) will be useful in treating results obtained with other self-associating systems, especially those involving a highly charged monomer where improved estimates of electrostatic effects are required as well as covolume considerations.

Appendix

Evaluation of the constant coefficients $\{\alpha_{ij}\}$ and $\{\alpha_{ijk}\}$

These sets of coefficients, corresponding respectively to second and third virial coefficients, are defined in eq. (3) and comment is now required on relations used in their numerical evaluation (tables 1 and 2). First, it is noted that the osmotic pressure, Π, of a solution comprising a self-associating protein and solvent may be expressed by the virial expansion,

$$(\Pi/RT) = \sum_i m_i + \sum_i \sum_j A_{ij} m_i m_j + \sum_i \sum_j \sum_k A_{ijk} m_i m_j m_k + \dots , \tag{A.1}$$

where the symbol A denotes an osmotic virial coefficient with concentrations expressed on the molar scale and the use of subscripts is the same as that employed in relation to eq. (3). Eqs. (3) and (A.1) together with the Gibbs Duhem relation may be used to show that

$$\alpha_{ij} = 2A_{ij} - M_j\bar{v} , \quad \alpha_{ijk} = (3A_{ijk}/2) - \bar{v}(M_j A_{ik} + M_k A_{ij})/2 , \tag{A.2), (A.3}$$

where $\bar{v}$ denotes the partial specific volume (liter/g) assumed identical for all oligomeric species. These relations are analogous to those obtained for a single solute [18] and for a self-associating system [7] in which activity coefficients were expressed as functions of total concentration rather than of solution composition as in eq. (3). It follows from eqs. (A.2) and (A.3) that values of α_{ij} and α_{ijk} may be computed if the corresponding osmotic virial coefficients are estimated on a statistical mechanical basis.

The coefficient A_{ij} in liter/mole may be calculated provided the potential energy $U_{ij}(x)$ of two protein molecules i and j is specified as a function of their center to center separation, x, in decimeters [35,36]. Thus,

$$A_{ij} = -2\pi N \int_0^\infty x^2 f_{ij}(x)\, dx , \tag{A.4}$$

where N is Avogadro's number and the "Mayer f-functions" are given by [36],

$$f_{ij}(x) = \exp(-U_{ij}(x)/kT) - 1 . \tag{A.5}$$

There are two major contributions to the potential energy to be considered, covolume effects and electrostatic interactions. It will be assumed that all oligomeric species are impenetrable spheres with radii r_i (i = 1, monomer; etc.) bearing a net charge Z_i on their surface, such that [16],

$$U_{ij}(x) = \begin{cases} \infty & \text{for } x \leqslant r_i + r_j \\ \dfrac{Z_i Z_j e^2 \exp\{-\kappa(x - r_i - r_j)\}}{\epsilon x(1 + \kappa r_i)(1 + \kappa r_j)} & \text{for } x > r_i + r_j \end{cases} \tag{A.6}$$

where e is the electronic charge, ϵ is the dielectric constant of the medium and κ is the inverse screening length of the supporting electrolyte given from Debye–Hückel theory in terms of the molar ionic strength, I, by $(8\,N\pi e^2\,I/1000\,\epsilon\,kT)^{1/2}$. Eq. (A.6) reduces to that cited by Phillies [37] and Tung and Steiner [38] for identical molecules, $i = j$. Eq. (A.5) may be written in expanded form as,

$$f_{ij}(x) = \begin{cases} -1 & \text{for } x \leqslant r_i + r_j \\ -(U_{ij}(x)/kT) + (U_{ij}(x)/kT)^2/2 - \ldots & \text{for } x > r_i + r_j \end{cases} \tag{A.7}$$

and, thus, eq. (A.4) becomes

$$A_{ij} = 2\pi N \int_0^{r_i + r_j} x^2\,dx - 2\pi N \int_{r_i + r_j}^{\infty} x^2 f_{ij}(x)\,dx\,. \tag{A.8}$$

The first integration may be performed directly to yield the contribution of the covolume effect [13,39,40]: the second integral may also be written explicitly provided the electrostatic energy satisfies the condition $U_{ij}(x) \ll kT$ for all $x > r_i + r_j$, so that the series in eq. (A.7) may be truncated after the first term. In this case, with the use of eq. (A.6), eq. (A.8) becomes

$$A_{ij} = \frac{2\pi N(r_i + r_j)^3}{3} + \frac{Z_i Z_j (1 + \kappa r_i + \kappa r_j)}{4I(1 + \kappa r_i)(1 + \kappa r_j)}\,. \tag{A.9}$$

In the limit that both κr_i and κr_j are small compared to unity (the Debye–Hückel limit) the second term of eq. (A.9) predicts a contribution to α_{ij} (via eq. (A.2)) of $Z_i Z_j/2I$ which is the term conventionally added to the covolume contribution [2,13,14,17]. However, use of this simplified correction in studies of charged proteins in buffers of ionic strength approximately 0.1 M may seriously overestimate the electrostatic contribution. For example, in this study, the value of I was 0.15 M and $T = 15°C$, so that $\kappa = 1.28\ \text{nm}^{-1}$ and thus even for the lysozyme monomer–monomer interaction ($i = j = 1$, $r_1 \sim 1.68$ nm), the coefficient of $Z_i Z_j/4I$ in eq. (A.9) is 0.53. It follows that eq. (A.9) is a better approximation for the calculation of the A_{ij} provided $U_{ij}(x) \ll kT$ for all $x > r_i + r_j$, a condition which depends on the magnitudes of Z_i and Z_j and may be tested with the use of eq. (A.6).

In the case of lysozyme at pH 8.0 where $Z_1 = +7.3$ [11], the latter condition was not fulfilled and, accordingly, an improvement on the second term of eq. (A.9) was sought. This was achieved by computing values of $x^2 f_{ij}(x)$ from eq. (A.5) as a function of x which permitted numerical evaluation of the integral shown as the second term in eq. (A.8). The required values of $U_{ij}(x)$ for this use of eq. (A.5) were computed using either eq. (A.6) or eq. (21) of ref. [41] with $\psi_{0i} = Z_i e/\epsilon r_i(1 + \kappa r_i)$ [42]. Plots of $U_{ij}(x)$ versus x from these two calculations intersect at a particular value of x: below this value, values of $U_{ij}(x)$ found on the basis of ref. [41] were used and, above it, those found from eq. (A.6) were employed. It is noted that eq. (21) of ref. [41] refers to the electrostatic potential between dissimilar spherical molecules i and j and is a generalized form of the equivalent expression for identical molecules ($i = j$) originally discussed by Derjaguin [15] and Verwey and Overbeek [16], embodying the DLVO theory.

It remains to discuss the evaluation of the A_{ijk} used in eq. (A.3) to calculate the α_{ijk}: the quantities A_{ik} and A_{ij} also contained in eq. (A.3) were computed as described above. Hill [40] has provided the following relation between A_{ijk} and the "Mayer f-functions" defined by eq. (A.5)

$$A_{ijk} = \frac{-8\pi^2 N^2}{3} \int x_{ij} f_{ij}(x_{ij})\,dx_{ij} \int x_{jk} f_{jk}(x_{jk})\,dx_{jk} \int x_{ik} f_{ik}(x_{ik})\,dx_{ik}\,, \tag{A.10}$$

where the limits of the first two integrals are taken from 0 to ∞ and of the third from $|x_{ij} - x_{jk}|$ to $x_{ij} + x_{jk}$. In the evaluation of A_{ijk}, values of f_{ij}, f_{jk} and f_{ik} as functions of x_{ij}, x_{jk} and x_{ik}, respectively, were found independently as described above. The triple integrations were then performed numerically according to a standard summation procedure.

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