Biophysical Chemistry 3 (1975) 169–176

THE DIRECT ANALYSIS OF SEDIMENTATION EQUILIBRIUM RESULTS OBTAINED WITH POLYMERIZING SYSTEMS

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Received 9 December 1974
Revised manuscript received 24 January 1975

Theory is presented in relation to sedimentation equilibrium results obtained with polymerizing systems, which permits evaluation of the activity of the monomer as a function of total weight concentration. In contrast to established methods, the suggested procedure does not involve the solution of simultaneous equations which are sums of exponentials or the determination of weight-average molecular weights. A major advantage of the method is that it avoids errors inherent in differentiation and integration steps. An extrapolation to infinite dilution is involved, but this is to a defined limit and is uncomplicated by the existence of critical points in the relevant plot. The method is capable of detecting possible volume changes inherent on polymer formation, of treating systems where activity coefficients of solute species are functions of total concentration and of describing the system in terms of relevant equilibrium constants. These points and comparisons with existing methods of analysis are illustrated with numerical examples and with results obtained with lysozyme at pH 6.7. The lysozyme results are interpretable in terms of either a non-ideal monomer–dimer system or a monomer–dimer–trimer system.

1. Introduction

In 1952 Steiner [1] outlined a method by which experimental results obtained with a polymerizing system, in the form of weight-average molecular weights as a function of total concentration, could be analysed to give the weight fraction of monomer at each total concentration. This procedure has been used extensively in the analysis of sedimentation equilibrium results obtained with polymerizing protein and low molecular weight ligand systems and has been extended to permit consideration of solutes whose activity coefficients are functions of total concentration [2–6]. It is noteworthy that the original treatment of Steiner [1] was formulated in a context where weight-average molecular weights had been obtained from light-scattering studies. In order to obtain similar data from sedimentation equilibrium experiments, a differentiation step is required [7,8] and when it is recognized that the Steiner procedure involves a numerical integration step, it becomes apparent that its application to sedimentation equilibrium results is somewhat circuitous and introduces error in both steps.

The aim of the present work is to present a procedure for the direct analysis of sedimentation equilibrium distributions obtained with either Rayleigh interference or absorption optics without recourse to differentiation or integration. The advantages of the method over established procedures are explored by use of computer-simulated examples and results obtained with the polymerizing lysozyme system.

2. Theory

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

$$\bar{c} = \sum_i c_i = \sum_i \psi_i c_1^i, \qquad (1)$$

where c_i is the weight concentration of species i of molecular weight iM_1 and ψ_i is the product of i equilib-

rium constants on the same concentration scale ($i = 1$, monomer; $\psi_1 = 1$ for reason of nomenclature). In general, the equilibrium constant $(K_i)_{j,k}$ may be defined as,

$$(K_i)_{j,k} = c_i/c_j c_k = \psi_i/\psi_j\psi_k; \qquad j + k = i, \tag{2}$$

where it is assumed that the ratio of the activity coefficients $y_i/y_j y_k$ equals unity [7]. For a successive polymerization $\psi_i = \Pi^i(K_I)_{1,I-1}$ and thus for tetramer formation, for example, $\psi_4 = (K_4)_{1,3}(K_3)_{1,2}(K_2)_{1,1}$ with $(K_1)_{1,0} = 1$: alternatively, $\psi_4 = (K_4)_{2,2}(K_2)^2_{1,1}$ where in general $(K_4)_{1,3} \neq (K_4)_{2,2}$. The latter formulation is particularly useful when ψ_3 is zero within the limits of detectability, whereupon values of ψ_2 and ψ_4 may be used to define the system operationally in terms of $(K_4)_{2,2}$ and $(K_2)_{1,1}$ appropriate to this case.

While eq. (1) requires for the elucidation of the equilibrium constants governing a polymerizing system that c_1 be found as a function of $\bar{c}$, it would be even more desirable to find the thermodynamic parameter a_1, the activity of the monomer, as a function of $\bar{c}$, for then non-ideality effects could also be considered. The Steiner procedure [1] does in fact yield the apparent weight fraction of monomer at various $\bar{c}$ values; but it does not make full use of two features of sedimentation equilibrium experiments which are not shared by light-scattering experiments. In sedimentation equilibrium studies a range of total concentration is spanned in any given experiment and there exists an exact relation between the activities of a given species at the different total concentrations encountered. Thus, the distribution at sedimentation equilibrium of each species i, in terms of its activity a_i, is given by [9],

$$a_i(r) = a_i(r_F)\exp i\phi_i M_1(r^2 - r_F^2), \tag{3a}$$

$$\phi_i = (1 - \bar{v}_i\rho)\omega^2/2RT, \tag{3b}$$

where r and r_F are any radial distances between or at r_m and r_b, the meniscus and base of the cell, respectively; ω is the angular velocity, $\bar{v}_i$ the partial specific volume of species i, ρ the solution density, R the gas constant and T the temperature of the sedimentation equilibrium experiment. Eq. (3a) may be written for monomer in terms of its weight fraction, $x_1(r) = c_1(r)/\bar{c}(r)$, as follows,

$$y_1(r)x_1(r)\bar{c}(r) = y_1(r_F)x_1(r_F)\bar{c}(r_F)\exp\phi_1 M_1(r^2 - r_F^2). \tag{4}$$

It is therefore possible to define an experimentally determinable dimensionless function, $\Omega(r)$, as

$$\Omega(r) = \frac{\bar{c}(r)\exp\phi_1 M_1(r_F^2 - r^2)}{\bar{c}(r_F)} = \frac{y_1(r_F)x_1(r_F)}{y_1(r)x_1(r)}. \tag{5}$$

Provided M_1 and $\bar{v}_1$ are known and any reference radial distance, r_F, is chosen to lie between or at r_m and r_b, it is a simple matter to construct a plot of $\Omega(r)$ versus $\bar{c}(r)$ from the experimental result obtained with Rayleigh interference or absorption optics without recourse to differentiation.

Comment is now required on the extrapolation of the plot of $\Omega(r)$ versus $\bar{c}(r)$ to infinite dilution. First, it follows from eq. (5) that,

$$\lim_{\bar{c}(r)\to 0} \Omega(r) = y_1(r_F)x_1(r_F) = a_1(r_F)/\bar{c}(r_F) \tag{6a}$$

since as $\bar{c}(r) \to 0$, $y_1(r) \to 1$ and $x_1(r) \to 1$. The latter limit follows from application of l'Hôpital's rule,

$$\lim_{\bar{c}(r)\to 0} x_1(r) = \lim_{\bar{c}(r)\to 0} dc_1(r)/d\bar{c}(r), \tag{6b}$$

where from eq. (1),

$$dc_1(r)/d\bar{c}(r) = \left\{1 + \sum_{i=2}^{i} i\psi_i(r)c_1^{i-1}(r) + \sum_{i=2}^{i} c_1^i d\psi_i(r)/dc_1(r)\right\}^{-1}. \tag{6c}$$

In formulating eq. (6c) it is noted that the ψ_i will be a function of r if volume changes accompany polymer formation, but in any event $d\psi_i(r)/dc_1(r) = 0$. Secondly, it follows from eq. (5) that,

$$d\Omega(r)/d\bar{c}(r) = y_1(r_F)x_1(r_F)[1 - d\ln a_1(r)/d\ln\bar{c}(r)]/a_1(r) \tag{7}$$

and therefore, that $d\Omega(r)/d\bar{c}(r) = 0$ only when $a_1(r)$ is a linear function of $\bar{c}(r)$. This shows that a critical point cannot exist in a plot of $\Omega(r)$ versus $\bar{c}(r)$, since from eq. (1) $a_1(r)$ can only be a linear function of $\bar{c}(r)$ when non-ideality and chemical interaction effects exactly compensate to mimic the behaviour of a single ideal solute ($\Omega(r) = 1$ for all values of $\bar{c}(r)$). It

will be shown later that the present analysis procedure offers advantage in this respect over the Steiner extrapolation [1] with certain systems. Thirdly, in particular cases where $c_1(r)$ may be written as an explicit function of $\bar{c}(r)$, an analytical relationship between $\Omega(r)$ and $\bar{c}(r)$ may be formulated and used to guide the extrapolation. Consider, for example, a monomer–dimer system with $y_1(r) = y_2(r) = 1$ and $\psi_2 = (K_2)_{1,1}$ independent of r (no volume change on reaction). It follows from eqs. (1) and (5) that,

$$\frac{1}{\Omega(r)} = \frac{(1+4(K_2)_{1,1}\bar{c}(r))^{1/2}-1}{2(K_2)_{1,1}\bar{c}(r)x_1(r_F)} \tag{8a}$$

$$\approx [1-(K_2)_{1,1}\bar{c}(r)+2(K_2)_{1,1}^2\bar{c}^2(r) - 5(K_2)_{1,1}^3\bar{c}^3(r)+\cdots]/x_1(r_F) \tag{8b}$$

where the converging alternating power series $(16(K_2)_{1,1}^2\bar{c}^2(r) \leqslant 1)$ may readily be extended if required. In the limit as $\bar{c}(r) \to 0$, it is clear that eq. (8b) is consistent with eq. (6a) for this thermodynamically ideal case. Another example for which an analytical relation similar to eq. (8b) may be written is provided by systems undergoing an isodesmic indefinite self-association [10, 11]. It is noted, however, that even for cases where such an explicit relation cannot readily be formulated, extrapolation of $\Omega(r)$ values to infinite dilution remains possible, a point which will be illustrated later.

The basic point emerges from this treatment that by application of eq. (6a), a means has been provided of finding $a_1(r_F)$ from a plot of $\Omega(r)$: it is thus possible with the use of eqs. (3) or (5) to evaluate $a_1(r)$ as a function of $\bar{c}(r)$. In this connection, it is noted that the value of $\bar{c}(r_F)$ used to compute $\Omega(r)$ from eq. (5) may be selected as any value in the observed total concentration range: this lack of restriction permits the construction of several $\Omega(r)$ versus $\bar{c}(r)$ plots each of which may be used to obtain estimates of the activity of the monomer at every total concentration. An averaging procedure is thereby suggested to obtain an improved plot of $a_1(r)$ versus $\bar{c}(r)$. Thus far, the analysis of a single sedimentation equilibrium experiment has been considered. However, the potential exists of conducting several experiments with varying initial concentrations or angular velocities and thereby of determining the activity of the monomer over a wide total concentration range, which may be important in distinguishing between different association models. Consider a system uncomplicated by volume changes $[(\Delta V_i)_{j,k} = (j+k)M_1\bar{v}_i - jM_1\bar{v}_j - kM_1\bar{v}_k = 0]$, on which two experiments have been performed such that a portion of the total concentration ranges which they encompass in their equilibrium distributions is common. Any value of $\bar{c}(r_F)$ is selected common to both experiments: this necessarily arises at different r_F positions. Eq. (5) is applied to obtain plots of $\Omega(r)$ versus $\bar{c}(r)$ relevant to each experiment, whereupon extrapolation yields $y_1(r_F)x_1(r_F)$ for each [eq. (6a)]. In this connection $y_1(r_F)$ is identical for each experiment because $\bar{c}(r_F)$ is common and similarly $x_1(r_F)$ must also be the same since the weight fraction of monomer is solely a function of the variable $\bar{c}(r)$, the equilibrium constants being pressure independent. It follows that plots of $\Omega(r)$ versus $\bar{c}(r)$ must be coincident in their common total concentration range.

On the basis of the extrapolated value of this coincident plot, the activity of the monomer at the reference concentration may be calculated [eq. (6a)] and hence values of $a_1(r)$ are available for the entire concentration range encompassed by both experiments. To aid the required extrapolation, it would be desirable to include in the set of two experiments one which was of the meniscus-depletion design so that the lowest measurable $\bar{c}(r)$ was included in the analysis. Consider now a third experiment which encompasses a higher total concentration range part of which is common to the range encountered in the first two experiments. Since the activity of monomer is known for each value of $\bar{c}(r)$ in the common range and hence for each corresponding value of r in the third experiment, application of eq. (3) yields $a_1(r)$ as a function of r and hence of the $\bar{c}(r)$ encountered in the third experiment. Evidently, this procedure may be extended to cover any desired range of total concentration.

It remains to consider the analysis of plots of $a_1(r)$ versus $\bar{c}(r)$ for the following relevant cases:

(i) All $y_i = 1$ and all $(\Delta V_i)_{j,k} = 0$. In this case corresponding values of $c_1(r)$ and $\bar{c}(r)$ are obtained directly and the relation between them is given by eq. (1). The coefficients ψ_i in this polynomial are constants and may be evaluated either by least-square regression or by the method described by Steiner [1] in

his treatment of equivalent data gained from the weight fraction of monomer at various total concentrations. The advantage of the present method then is the avoidance of differentiation and subsequent numerical integration in obtaining the weight fraction results rather than in their analysis to yield the ψ_i. Once values of ψ_i have been obtained, application of eq. (2) gives the values of $(K_i)_{j,k}$ suitable for the description of the system.

(ii) All $y_i \neq 1$ and all $(\Delta V_i)_{j,k} = 0$. One simple approach to the analysis of $a_1(r)$ versus $\bar{c}(r)$ results obtained with systems of this kind involves assuming that $\ln y_i(r) = iBM_1\bar{c}(r)$ [4,7], the first term of a power series. This assumption is consistent with the previously stipulated requirement that $y_i(r)/y_j(r)y_k(r) = 1$ and introduces the additional specification that all activity coefficients may be described in terms of a single non-ideality coefficient, B, which is taken to be concentration independent. Since $c_1(r) = a_1(r)/\exp BM_1\bar{c}(r)$, the available results may be analysed as before employing eq. (1) for an assigned value of B, which may be successively refined on the basis that this polynomial must be strictly obeyed. In cases, however, where $c_1(r)$ may be written as an explicit function of $\bar{c}(r)$, an alternative procedure for the evaluation of the parameters is possible. As before, an instance is provided by a monomer–dimer system for which,

$$c_1(r) = \{(1+4(K_2)_{1,1}\bar{c}(r))^{1/2}-1\}/2(K_2)_{1,1}, \qquad (9a)$$

$$B = \ln\{a_1(r)/c_1(r)\}^{1/M_1\bar{c}(r)}. \qquad (9b)$$

Combination of eqs. (9a) and (9b) when written for two $(a_1(r), \bar{c}(r))$ points yields an expression in the single unknown $(K_2)_{1,1}$ which can be solved numerically: the value of B follows directly.

(iii) All $(\Delta V_i)_{j,k} \neq 0$. If volume changes are involved in the formation of higher polymers, the ψ_i in eq. (1) become functions of r. This does not invalidate the previously described method of determining the plot of $a_1(r)$ versus $\bar{c}(r)$ from a single sedimentation equilibrium distribution, since in eq. (6c) ψ_i has been considered to be a function of r. It is unlikely, however, that experimental precision would permit the evaluation from such data of several $(\Delta V_i)_{j,k}$ which are not necessarily related [13]. However, the operation of volume changes may in principle be detected by conducting two experiments employing different values

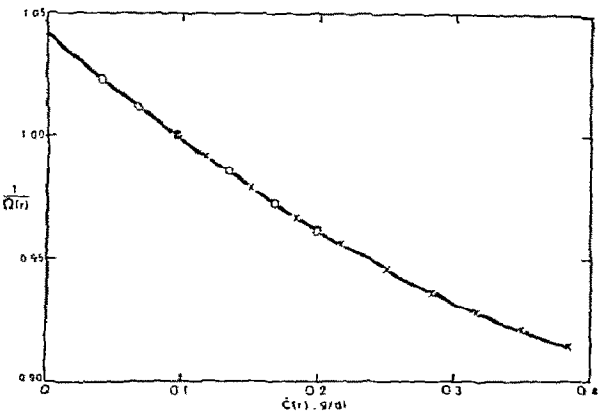

Fig. 1. Computer-simulated sedimentation equilibrium results obtained with a monomer–dimer system with initial loading concentrations of 0.2363 g/dl (×) and 0.0991 g/dl (○): other experimental parameters are cited in the text. The plot is one of $1/\Omega(r)$, defined by eq. (5), versus the total protein concentration, $\bar{c}(r)$. The solid line was computed from eq. (8b) with $x_1(r_F) = 0.962$ and $(K_2)_{1,1} = 0.44$ dl/g.

of ω and/or total loading concentration such that a portion of the total concentration ranges which they encompass in their equilibrium distributions is common, as before. In cases where all $(\Delta V_i)_{j,k} = 0$, it has been shown that plots of $\Omega(r)$ versus $\bar{c}(r)$ based on a common $\bar{c}(r_F)$ are coincident in their common total concentration range. In contrast, when the $(\Delta V_i)_{j,k} \neq 0$, a different value of $x_1(r_F)$ pertains to the separate experiments even though the same $\bar{c}(r_F)$ (and hence $y_1(r_F)$) has been selected. This arises as a consequence of the different values of the equilibrium constants due to the different pressures at the different r_F points. Therefore, in these cases, the plots of $\Omega(r)$ versus $\bar{c}(r)$ will intersect at the point $(\bar{c}(r_F), \Omega(r) = 1)$, but otherwise will not be coincident.

3. Numerical illustrations

In order to illustrate the application of eqs. (5) and (6a), sedimentation equilibrium distributions were simulated [14] for a thermodynamically ideal monomer–dimer system with $M_1 = 14\,400$, $(K_2)_{1,1} = 0.44$ dl/g and $\bar{v}_1 = \bar{v}_2 = 0.726$ ml/g, the values being chosen to resemble those pertaining to the lysozyme system at pH 6.7, ionic strength 0.17, 15°C [5,15].

Two experiments were considered both with ω = 20 000 rpm, T = 15°C, ρ = 1 g/ml, r_m = 6.9 cm and r_b = 7.2 cm, the initial loading concentrations being 0.2363 g/dl and 0.0991 g/dl. The simulated distributions of $\bar{c}(r)$ versus r both included the total concentration of 0.0936 g/dl which was chosen as $\bar{c}(r_F)$: it occurred at r_F values of 6.9 cm and 7.0608 cm in the experiments conducted with the higher and lower loading concentrations, respectively. Application of eq. (5) yielded $\Omega(r)$ values which are plotted in reciprocal form versus corresponding $\bar{c}(r)$ values in fig. 1. It is evident that the curves are coincident, a result in accord with the specification that $\bar{v}_1 = \bar{v}_2$ (no volume change). A similar computation with the same parameters except $(\Delta V_2)_{1,1}$, which was set as −860 ml/mole of dimer ($\bar{v}_1$ = 0.726 ml/g, $\bar{v}_2$ = 0.696 ml/g), confirmed that plots of $\Omega(r)$ versus $\bar{c}(r)$ based on a common $\bar{c}(r_F)$ value intersected at $\Omega(r) = 1$, but were non-coincident: it is noted, however, that the maximum percentage difference in $\Omega(r)$ values recorded at a common $\bar{c}(r)$ point was only 1%, which would be difficult but not impossible to detect in practice. The second noteworthy feature of fig. 1 is that extrapolation to $\bar{c}(r) \to 0$ is unambiguous and leads to a value of $x_1(r_F)$ of 0.962, in agreement with the theoretical value calculated from eq. (9a) with $\bar{c}(r_F)$ = 0.0936 g/dl. This value of $x_1(r_F)$ together with the chosen $(K_2)_{1,1}$ was used in eq. (8b) to construct the theoretical solid line in fig. 1. It is evident that eq. (8b) could have been used to guide the extrapolation in an experimental context. Moreover, the fit of the solid line to the points shown suffices to illustrate the consistency of the analysis procedure, which was also evident by the fit of the derived plot $c_1(r)$ versus $\bar{c}(r)$ to eq. (1).

It would be unfortunate to create the impression that the basic extrapolation is as direct as is shown in fig. 1 for all systems. Fig. 2A illustrates this point in relation to a thermodynamically ideal monomer–dimer–trimer system with M_1 = 14 400, $\bar{v}_1 = \bar{v}_2 = \bar{v}_3$ = 0.73 ml/g, $(K_2)_{1,1}$ = 0.1 dl/g, $(K_3)_{1,2}$ = 10 dl/g. Although the curves from two different equilibrium experiments conducted with different initial loading concentrations overlap, extrapolation to the correct value of $x_1(r_F)$ of 0.958 ($\bar{c}(r_F)$ = 0.1732 g/dl) is primarily guided by the results obtained with the lower loading concentration. It is instructive, however, to compare this extrapolation with that required in the

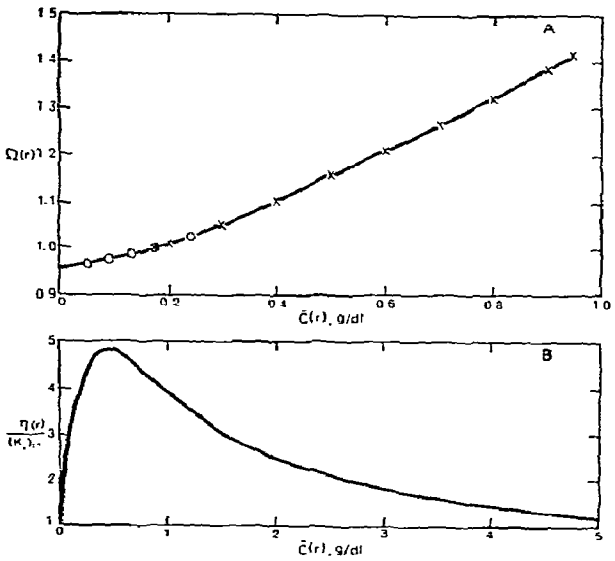

Fig. 2. Sedimentation equilibrium results computed for the monomer–dimer–trimer system specified in the text. (A) The results plotted in the form of the dimensionless function $\Omega(r)$, eq. (5), versus the total protein concentration. The sedimentation parameters selected were as follows: ω = 20 000 rpm, T = 15°C, ρ = 1 g/ml, r_m = 6.9 cm, r_b = 7.2 cm and initial loading concentrations of 0.4953 g/dl (×) and 0.1206 g/dl (○). The solid line is an attempt to average the data and indicates the extrapolation to infinite dilution. (B) $\eta(r)$, a function of the weight-average molecular weight for the same system, plotted in normalized form as $-\eta(r)/(K_2)_{1,1}$ versus the total protein concentration according to the basic formulation suggested by Steiner [1]. For details see text.

analysis of sedimentation equilibrium results relevant to the same system by the Steiner procedure [1]. Experimentally, this requires evaluation of apparent weight-average molecular weight values by differentiating a plot of $\ln\bar{c}(r)$ versus r^2: in practice error is introduced in this operation, but this is obviated in the present numerical example by calculating M_{wr} as a function of $\bar{c}(r)$ directly. Steiner suggested that a plot be constructed of $\eta(r) = [(M_1/M_{wr})-1]/\bar{c}(r)$ versus $\bar{c}(r)$, since the area under this plot between $\bar{c}(r) = 0$ and $\bar{c}(r)$ gives $\ln\{x_1(r)\}$. The procedure, therefore, requires extrapolation of experimentally determined values of $\eta(r)$ to $\bar{c}(r) \to 0$. Fig. 2B presents a plot of $-\eta(r)/(K_2)_{1,1}$ versus $\bar{c}(r)$, the ordinate being normalized by noting that application of l'Hôpital's rule shows that $\lim_{\bar{c}(r)\to 0} \eta(r) = -(K_2)_{1,1}$. The inter-

esting feature of fig. 2B is the appearance of a maximum, which renders hazardous the extrapolation required for this system since the value of $(K_2)_{1,1}$ is unknown to the experimenter. More generally it may be shown by application of Descarte's rule that a critical point will exist in the plot for a monomer–dimer–trimer system if $3(K_2)_{1,1} < 2(K_3)_{1,2}$. This relation follows from integrating the solution of $d\eta(r)/d\bar{c}(r) = 0$ to obtain

$$M_{wr}\bar{c}(r) = e^{\beta}(M_{wr} - M_1), \qquad (10)$$

where e^{β} is the integration constant. Eq. (10) applies to all systems and may be rewritten for particular systems as a polynomial in $c_1(r)$. For the monomer–dimer–trimer system under discussion, the polynomial was examined by Descarte's rule to define the conditions for the existence of two positive and real roots, one defining e^{β} which is necessarily positive and the other pertaining to the critical point. It follows that with certain systems (e.g. $(K_2)_{1,1} > \frac{2}{3}(K_3)_{1,2}$) the Steiner extrapolation offers little difficulty; but with others it is to be avoided. In this connection, it is restressed that a plot of $\Omega(r)$ versus $\bar{c}(r)$ cannot exhibit a critical point for any system.

Another example of a type of system for which extrapolation of this plot may prove difficult is one in which the association constants are sufficiently large so that the weight-fraction of the monomer at the reference position is small. In this instance and in contrast to the behaviour shown in fig. 2A, the $\Omega(r)$ versus r plot would tend to a small number as $\bar{c}(r) \to 0$ with steep slope. It is recognised that the analysis of this type of system by established procedures is exceedingly difficult; but it may prove feasible by the present direct method, provided experimental data of the required precision are available from a meniscus-depletion experiment and $\bar{c}(r_F)$ is selected as low as is possible so that $x_1(r_F)$ approaches as close as is possible its maximum value of unity.

4. Experimental

Salt-free lysozyme (hens' egg white) was obtained from Worthington Biochemical Corp. Solutions for sedimentation equilibrium experiments were prepared by dissolving an appropriate amount of the protein in a buffer of pH 6.7, ionic strength 0.17 (0.005 M sodium dihydrogen phosphate, 0.005 M disodium hydrogen phosphate, 0.15 M sodium chloride) and dialysing exhaustively at room temperature against approximately 25 volumes of the buffer in washed Visking 8/32 cellophane tubing.

Sedimentation equilibrium experiments were performed at 20 000 rpm and 15°C with a Spinco model E ultracentrifuge fitted with electronic speed control and a Rayleigh interference optical system. The procedures for the conduct of the experiments, determination of initial loading concentrations (0.258 g/dl and 0.1025 g/dl in the present instance), and the measurement of the interferograms to obtain plots of $\bar{c}(r)$ versus r at equilibrium have been described in detail previously [15,16]. The partial specific volume and monomeric molecular weight of lysozyme were taken as 0.726 ml/g [17] and 14 400 [5,15], respectively. Protein concentrations originally determined in Rayleigh interference fringes were converted to their equivalent values in g/dl by measuring the optical density at 280 nm of a solution whose concentration in Rayleigh interference fringes was also measured in a synthetic boundary cell. The use of an extinction coefficient $E^{1\%}_{1\,cm}$ of 26.35 [18] led to the relationship $c = 0.0254\,J$ where c is in g/dl and J in Rayleigh interference fringes in a 12 mm path length ultracentrifuge cell.

5. Results

The two concentration distributions ($\bar{c}(r)$ versus r) obtained in the sedimentation equilibrium experiments were converted to the plots of $\Omega(r)$ versus $\bar{c}(r)$ [eq. (5)] shown in fig. 3A. The value of 0.15 g/dl, common to both distributions, was chosen as $\bar{c}(r_F)$, the corresponding values of r_F being 7.0975 cm and 6.9291 cm for the experiments conducted with the lower and higher loading concentrations, respectively. It is evident from fig. 3A that the values of $\Omega(r)$ derived from the two separate experiments are coincident over their common concentration range, showing in accordance with the results of previous studies [5, 15] that no detectable volume change accompanies the polymerization of lysozyme at pH 6.7. The extrapolation of $\Omega(r)$ to $\bar{c}(r) \to 0$ shown in fig. 3A yielded a value of $y_1(r_F)x_1(r_F)$ of 0.9395 and hence of 0.1409 for $a_1(r_F)$. This value was employed in eq. (3) to calculate $a_1(r)$

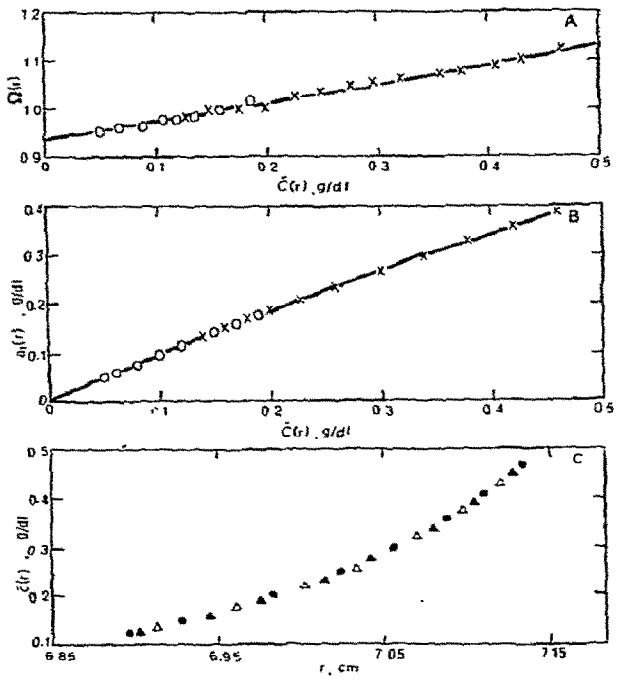

Fig. 3. Experimental sedimentation equilibrium results obtained at 20 000 rpm and 15°C with lysozyme in buffer of pH 6.7, ionic strength 0.17. (A) The plot corresponding to fig. 2A with initial loading concentrations of 0.258 g/dl (×) and 0.1025 g/dl (○). In each experiment the column height was 0.28 cm with $r_b = 7.1468$ cm. The solid line indicates the extrapolation to $y_1(r_F)x_1(r_F) = 0.9395$. (B) The corresponding plot of $a_1(r)$ versus $\bar{c}(r)$, based on the extrapolated infinite dilution value found from fig. 3A and on eq. (3). (C) A comparison of the experimentally observed points $(\bar{c}(r), r)$ shown as ●, found in the experiment conducted with an initial loading concentration of 0.258 g/dl with data simulated for the following systems: △, a monomer–dimer system with $(K_2)_{1,1} = 0.44$ dl/g and $BM_1 = -0.02$ dl/g; ▲, a monomer–dimer–trimer system with $(K_2)_{1,1} = 0.46$ dl/g, $(K_3)_{1,2} = 0.08$ dl/g and $BM_1 = 0$.

as a function of r and hence of $\bar{c}(r)$, results which are shown in fig. 3B. The plot passes through the origin indicating that no refinement of the extrapolated $y_1(r_F)x_1(r_F)$ value was required in this case.

The results shown in fig. 3B were analysed on the basis of two models. First, it was assumed following previous findings that the system was non-ideal with $BM_1 = -0.02$ dl/g [5,15], which permitted transformation of the ordinate values in fig. 3B to corresponding values of $c_1(r)$ by division by $\exp(BM_1\bar{c}(r))$. Application of eq. (1) led to a least-square estimate of $\psi_2 = (K_2)_{1,1} = 0.44 \pm 0.01$ dl/g, a t-test showing that terms in the polynomial of higher order than two were not significant. These calculations show, therefore, that the previous interpretation in terms of a non-ideal monomer–dimer system is consistent with the experimental results analysed by the present procedure. This consistency is further emphasized in fig. 3C where the experimental points are compared with those found by computing the distribution with $(K_2)_{1,1} = 0.44$ dl/g and $BM_1 = -0.02$ dl/g. The second model which was considered involved setting $BM_1 = 0$, implying that the ordinate values in fig. 3B are those of $c_1(r)$. In this case, fitting of eq. (1) gave least-square estimates of ψ_2 and ψ_3 corresponding to $(K_2)_{1,1} = 0.46 \pm 0.01$ dl/g and $(K_3)_{1,2} = 0.08 \pm 0.06$ dl/g [eq. (2)]: application of the t-test showed that tetramers and higher polymers were not present in significant amounts. The values of $\bar{c}(r)$ versus r computer-simulated for this second model are shown in fig. 3C and it is seen that they also satisfactorily describe the basic experimental results.

6. Discussion

In relation to the particular lysozyme system which has been re-examined in this study, comment need only be made on the alternative interpretations, which both describe the experimental results (fig. 3C). The concept that a negative virial coefficient applies to monomer and dimer has previously been questioned [19], and indeed it is somewhat surprising in studies conducted at relatively high ionic strength where molecular covolume contributions might be expected to dominate the magnitudes of the virial coefficients [20]. It is, therefore, of interest that the results are also in accord with a monomer–dimer–trimer system, especially as lysozyme is thought to polymerize via a 'head-to-tail' mechanism [19,21,22] and polymers greater than the dimer are known to exist in appreciable amounts at more alkaline pH values closer to the isoelectric point [23].

In a more general sense, it was the major purpose of this work to present a method of obtaining $a_1(r)$ as a function of $\bar{c}(r)$ from sedimentation equilibrium results. It is relevant, therefore, to summarize the ad-

vantages of the present procedure over established methods of analysis. Several workers [3,9,24] have discussed a method for analysing sedimentation equilibrium results involving solution of a set of linear simultaneous equations describing $\bar{c}(r)$ at selected r values. The procedure therefore also utilizes $\bar{c}(r)$ versus r results directly, but is difficult to apply when complications due to non-ideality effects or volume changes are encountered, rendering the equations non-linear. Moreover, the method requires an initial selection of a model specifying the species coexisting in equilibrium and in this respect is at a disadvantage to methods which lead to plots of $x_1(r)$ or $a_1(r)$ versus $\bar{c}(r)$ without such a specification. The use of a plot of $\eta(r)$, a function of apparent weight-average molecular weights, versus $\bar{c}(r)$ leads to such a formulation in terms of the apparent weight fraction of monomer [1,3,4]. However, it suffers from three disadvantages in comparison with the method described in this work. First, a differentiation step is involved in the evaluation of apparent weight-average molecular weights and although several procedures have been devised to minimize error, it would be preferable to avoid this step completely. Secondly, the extrapolation of $\eta(r)$ to $\bar{c}(r) \to 0$ is not without hazard for certain systems (fig. 2B). Thirdly, following the extrapolation, it is required to perform a numerical integration to obtain the final result, a step introducing further error: an additional numerical integration is required if apparent number-average molecular weights are to be derived from sedimentation equilibrium results and used in the estimation of activity coefficients [4,25]. The significant feature of the present analysis is that it leads directly to information on the activity of the monomer as a function of total concentration, and requires neither integration nor differentiation steps but only extrapolation of a function ($\Omega(r)$) to a defined limit without complication due to the existence of critical points in the relevant plot.

It is hoped that the analysis procedure outlined herein will assist in the elucidation of a diversity of polymerizing systems in terms of the detection of possible volume changes if these are sufficiently large, the treatment of non-ideality effects and the estimation of relevant equilibrium constants describing the systems.

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