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Osmotic pressure measurements of ovalbumin and lysozyme mixtures

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Ovalbumin and lysozyme have been reported to undergo a mixed association in solutions of low ionic strength. Osmotic pressure experiments were performed on ovalbumin and on lysozyme solutions in 0.06 M cacodylate buffer ($I = 0.02$, pH = 5.8) at 30 and at 37 °C. The individual proteins did not undergo any self-associations at either temperature; these measurements indicated that each of the solutions was nonideal. Osmotic pressure experiments on three blends of lysozyme and ovalbumin at 30 and 37 °C could be interpreted in two ways. One interpretation was that a nonideal, nonassociating mixture of A and B was present; for the three solutions the mixed nonideal term B_{AB} was negative. A negative nonideal term is usually interpreted as indicating an association. The other interpretation of the data was as a quasi-ideal mixed association of the type $A + B \rightleftarrows AB$.

1. Introduction

Associations involving two different reactants, such as

$$\left.\begin{array}{l} A + B \rightleftarrows AB, \\ n_A \rightleftarrows A_n,\ n = 2, 3, \ldots \\ A + B \rightleftarrows AB \end{array}\right\}$$

and related equilibra are known as mixed associations. These associations are quite important in biochemistry and biology. The antigen-antibody reaction is probably the best known example of a mixed association [1,2]. Other well-known examples are enzyme-enzyme inhibitor reactions [3–5], the interaction of serum albumin with hyaluronic acid [6,7], and the interaction between ovalbumin and myoglobin [8]. Recently, the interactions between mixtures of apolipoproteins A-II and C-I have been reported [9]. Mixed associations also occur with small molecules, such as the interactions in $CHCl_3$ solutions between cholesterol and methyl cholate [10] or between 1-cyclohexyluracil and 9-ethyladenine [11], and the association in aqueous solutions between purine and uridine [12].

With larger molecules like proteins, one can study mixed associations by techniques that measure average or apparent average molecular weights [13,14]. Light scattering measurements require knowledge of the refractive index increments of the reactants, and one must make an assumption about the refractive index increment of the complex or complexes [14]. In addition, one must be very careful to remove dust from the solutions. In sedimentation equilibrium experiments one has the problem of the refractive index increments when refractometric (Rayleigh or schlieren) optics are used. Partial specific volumes or density increments of the reactants must be known, and one must make appropriate assumptions about the value of these quantities for the complex or complexes. Furthermore, sedimentation equilibrium experiments take time, and the analysis may be

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complicated by the redistribution of the associating constituents in the ultracentrifuge cell [14].

The theory for membrane osmometry is much simpler [13]. While one needs to know the concentration of the solutes, it is not necessary to know values of the refractive index increments, partial specific volumes or density increments in order to perform the experiments. The technique is nondestructive, and the samples can be recovered. With the development of high-speed membrane osmometers, the measurement of osmotic pressure has become easier and more realistic. In addition, one can perform osmotic pressure experiments at higher temperatures than are generally possible in the ultracentrifuge. Thus, it is possible to perform experiments at 37° C and mimic physiological conditions.

Ovalbumin and lysozyme are readily available. At low ionic strength they have been reported to undergo mixed associations at 25° C according to results obtained from analytical gel chromatography and ultracentrifuge experiments [15–19]. We wished to study this system by membrane osmometry for the reasons given above. In addition studies of this kind can serve as a model for other heterogeneous protein-protein interactions.

2. Experimental

2.1. Materials

Recrystallized, lyophilized ovalbumin (Sigma grade V, 99% electrophoretically pure) and lysozyme (Sigma grade I, 3 times recrystallized, dialyzed) were used in these experiments. Cacodylic acid (dimethylarsenic acid, Schwarz/Mann) and reagent grade sodium cacodylate trihydrate (Fisher) were used as received.

The cacodylate buffer was prepared by dissolving 8.56 g (0.02 mol) sodium cacodylate trihydrate and 11.1 g (0.04 mol) cacodylic acid in redistilled water to a final volume of 1 l. At 25° C the buffer had a pH of 5.80 ± 0.02, ionic strength 0.02 and density 1.0013 ± 0.0002 g/ml. Protein solutions were prepared by dissolving approx. 2 g protein in 100 ml buffer. These solutions were dialyzed against 1 l of buffer using Spectrapor membrane tubing (retention ~ 6 kDa). Dialysis was carried out in the cold over a 3 day period with four changes of buffer. Following dialysis the solutions were passed through a 0.45 μm Millipore filter and stored in the cold (4–6° C) in sealed vials. Stock solutions remained clear and were used within 1 week.

Concentrations of the protein stock solutions were determined by differential refractometry (Brice-Phoenix model BP-2000-V) at 25° C and 546 nm. The refractive index difference Δn, of the dialyzed solution is related to the concentration c in g/l by

$$\Delta n = \psi c \tag{1}$$

The refractive index increments, ψ, were taken to be 1.88×10^{-4} l/g for ovalbumin [20] and 1.89×10^{-4} l/g for lysozyme [21,22].

To check the consistency of the concentration determinations from Δn values, we measured the absorbance of some diluted solutions of ovalbumin and of lysozyme at 280 nm in a dual-beam spectrophotometer (Cary model 219). From these measurements we calculated $E^{1\%}_{1\,cm}$, the absorptivity of 1% (1 g/dl) solution in a cuvette of thickness 1 cm. Our values for $E^{1\%}_{1\,cm}$ of 7.12 (ovalbumin) and 26.88 (lysozyme) compare favorably to the literature values of 7.14 [23], 7.15 [24] and 7.35 [25] for ovalbumin and 26.35 [26] and 27.2 [27] for lysozyme.

2.2. Osmometry

Osmotic pressure measurements were carried out at 30 and 37° C in a Knauer no. 01.00 membrane osmometer. Its operation is described in the manual provided with the instrument and is similar to procedures described previously [13]. The rapid equilibration time (< 20 min) of this osmometer is due to the large surface-to-volume ratio in the measuring cell. Pressure tracings were recorded using a Dohrman 11 inch chart recorder.

Schleicher and Schuell AC61 membranes (retention ~ 10 kDa) were used. These membranes were stored in the cold in a 25–30% (v/v) ethanol/water solution. Before use, the membranes were soaked for 1 h in water, then 1 h in the buffer solution. The buffer solution was changed,

the membrane was cut to size, and then the buffer solution and membrane were degassed for 15–30 min using a vacuum line or an aspirator to remove the air bubbles. The osmometer cell was rinsed several times with warm, degassed buffer, and the membrane was inserted carefully to avoid trapping bubbles. Generally, the antiballooning screen was not necessary. The membrane was clamped to the cell according to the instructions in the manual, and the stresses due to the installation of the membrane were allowed to settle overnight.

Although membrane osmometry is an absolute method, the instrument must be calibrated prior to each set of measurements. One can adjust the chart recorder using the appropriate potentiometers on the osmometer, so that a change in pressure of 10 cm corresponds to a full-scale deflection on the chart recorder. The 10 cm pressure change is brought about through the calibration device. This device, which contains solvent or buffer, is connected to the osmometer; when it is lowered from the upper to lower setting, the height is decreased by 10 cm. Calibration is performed with degassed buffer or solvent, and care must be taken to ensure that there are no bubbles in the connecting tubing or in the osmometer. The pen displacement for a 10 cm change in the hydrostatic pressure of the solvent was measured after 3 min setting time and averaged over three or more repetitive measurements.

Each solution was degassed prior to injection into the osmometer. Before measuring the osmotic pressure (Π) it is necessary to rinse the membrane at least three times with the appropriate protein solution. Four or five 400-μl injections of each protein solution were used for each measurement, and these were checked for reproducibility at 3–5 min setting time. Solutions were introduced in order of increasing concentration.

The solutions used for measuring the osmotic pressure of the individual proteins were prepared from dilution of the stock solution using buffer in dialysis equilibrium with it. In order to study mixtures of ovalbumin and lysozyme, it was necessary to prepare working stock solutions having a definite concentration ratio, β, of each reactant. Here $\beta = c_A^\circ/c_B^\circ$, where c_A° and c_B° are the initial concentrations of A and B in the blend (assuming no self-associations or mixed associations are occurring). One carries out a series of experiments at constant β by diluting the working stock solution with the dialysate.

Results

3.1. Individual protein solutions

For a nonassociating solute the osmotic pressure, Π, is related to the apparent molecular weight, M_{app}, by [13]

$$\frac{\Pi}{RT} = \frac{c}{M_{app}} = \frac{c}{M} + \frac{Bc^2}{2} \tag{2}$$

where Π is the osmotic pressure in atm, R the gas constant (0.08205 l atm K^{-1} mol^{-1}), T the absolute temperature, c the protein concentration in g/l, M the molecular weight and B the osmotic pressure second virial coefficient. Since the proteins were dialyzed against buffer, one can use the Casassa-Eisenberg [28] or Vrij-Overbeek [29] conventions for defining the macromolecular component, and the osmotic pressure equation becomes formally identical to that for a two-component system. The osmotic pressure in cm of solvent must be converted to atm before eq. 2 can be applied. Here, one notes that the conversion factor is

$$f = 76\left[\rho_{Hg}/\rho_{solvent}\right]_T \tag{3}$$

Thus

$$\Pi(\text{atm}) = \Pi(\text{cm solvent})/f \tag{4}$$

The solvent (buffer) density was 1.0013 ± 0.0002 g/ml at 25.8° C. We estimated the density of the solvent at other temperatures by assuming

$$\left[\rho_{T_2}/\rho_{T_1}\right]_{solvent} = \left[\rho_{T_2}/\rho_{T_1}\right]_{H_2O} \tag{5}$$

Fig. 1A shows a plot of Π/c vs. c for ovalbumin at 37° C (310.2 K), and fig. 1B gives the corresponding plots at 30° C (303.2 K). It is evident from these plots that there is no self-association. Fig. 1C and D shows the corresponding plots of Π/c vs. c for lysozyme. The values of M obtained at the two temperatures are listed in table 1, and those of B_{OS} are recorded in table 2.

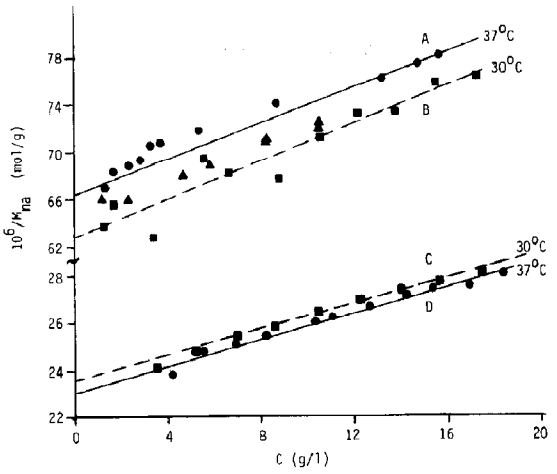

Fig. 1. Plots of Π/c vs. c for ovalbumin solutions at 37 °C (A) and 30 °C (B) in 0.06 M cacodylate buffer (0.2 ionic strength, pH 5.8). The corresponding plots for lysozyme are shown in traces C (37 °C) and D (30 °C). The inclined straight lines for these plots indicate that the solutions are nonideal and that no self-association is occurring. The different symbols in some of the plots indicate measurements made on different dialyzed stock solutions.

Table 2

Results for osmotic pressure second virial coefficients B_{OS}, with standard errors

Values computed by linear least-squares, using $B_{OS} = 2\Delta(\Pi/cRT)/\Delta c$.

Concentration ratio $\beta = c_A^\circ/c_B^\circ$	B_{OS} (1 mol g^{-2}) ($\times 10^6$)	
	$T = 310$ K	$T = 303$ K
∞ (ovalbumin)	0.57 ±0.03	0.54 ± 0.02
0 (lysozyme)	1.5 ±0.2	1.6 ±0.2
1.014	−0.48 ±0.2	–
0.532	0.081 ± 0.025	–
1.012	–	−0.44 ± 0.14

3.2. Protein mixtures

When studying a mixture of two proteins, it is necessary to prepare a working stock solution having a fixed concentration ratio β of the two proteins. Here $\beta = c_A^\circ/c_B^\circ$, where c_A° or c_B° is the concentration (in g/l) of A or B in the blend. One must carry out a series of osmotic pressure experiments at constant β; this is done by making dilutions of the working stock with the dialysate. If no mixed association occurs, then at constant β one notes that M_n is a constant; it is related to β by

Table 1

Results for limiting number-average molecular weights, M_n°, with standard errors, from linear least-squares extrapolations

Concentration ratio $\beta = c_A^\circ/c_B^\circ$	Number-average molecular weight M_n° (g/mol)	
	$T = 310$ K	$T = 303$ K
∞ (ovalbumin)	43400 ± 450	42500 ± 350
0 (lysozyme)	15100 ± 450	16000 ± 400
1.014	23350 ± 200 [a]	–
	22480 ± 560 [b]	
0.5319	19700 ± 100 [a]	–
	19520 ± 490 [b]	
1.012	–	22750 ± 200 [a]
		23310 ± 490 [b]

[a] Value from extrapolation of mixture data.

[b] Expected value from eq. 17, using $M_A = M_n^\circ$ ($\beta = \infty$) and $M_B = M_n^\circ$ ($\beta = 0$).

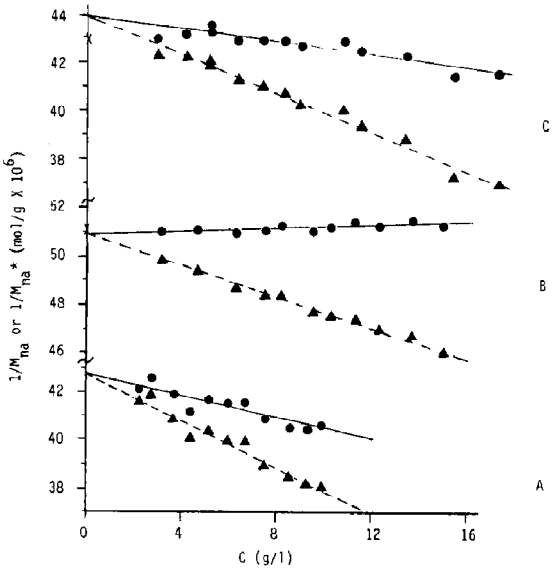

Fig. 2. Plots of Π/c vs. c for three ovalbumin-lysozyme blends in cacodylate buffer under various conditions: (A) 37 °C, $\beta = 1.014$; (B) 37 °C, $\beta = 0.532$; (C) 30 °C, $\beta = 1.012$. The solid line plots are based on eq. 11; they suggest that we are dealing with a nonideal mixture and that no mixed association is present. The dashed line plots are based on eq. 18.

$$M_n = \frac{c_A^o + c_B^o}{\frac{c_A^o}{M_A} + \frac{c_B^o}{M_B}} = \frac{1+\beta}{\frac{\beta}{M_A} + \frac{1}{M_B}} \quad (6)$$

since

$$\beta = c_A^o / c_B^o \quad (7)$$

and

$$c = c_A^o + c_B^o = c_B^o(1+\beta) = (c_A^o/\beta)(1+\beta) \quad (8)$$

If the solution is nonideal one notes that

$$\ln y_i = M_i \sum_j B_{ij} c_j \quad (9)$$

(i or j = A or B)

and

$$B_{ij} = B_{ji} \quad (10)$$

With these relations one obtains [13]

$$\frac{\Pi}{RT} = \frac{c}{M_{na}} = \frac{c}{M_n} + \tfrac{1}{2}\sum_i \sum_j B_{ij} c_i^o c_j^o$$
$$= \frac{c}{M_n} + \tfrac{1}{2}\left[B_{AA}(c_A^o)^2 + B_{BB}(c_B^o)^2 + 2B_{AB}c_A^o c_B^o\right]$$
$$= \frac{c}{M_n} + \left[\beta^2 B_{AA} + B_{BB} + 2\beta B_{AB}\right]\frac{c^2}{2(1+\beta)^2}$$
$$= \frac{c}{M_n} + \frac{B_{OS}c^2}{2} \quad (11)$$

Here

$$B_{OS} = \frac{1}{(1+\beta)^2}\left[\beta^2 B_{AA} + B_{BB} + 2\beta B_{AB}\right] \quad (12)$$

For the ideal case $B_{OS} = 0$.

If a mixed association such as

$$nA + mB \leftrightarrows A_nB_m \quad (n, m = 1, 2, \ldots) \quad (13)$$

occurs, then one uses eqs. 7 and 8; one also assumes that

$$\ln y_{A_nB_m} = n \ln y_A + m \ln y_B \quad (14)$$

At constant β, M_n becomes M_n^{eq}, which is now a function of c. The osmotic pressure equation becomes [13]

$$\frac{\Pi}{RT} = \frac{c}{M_{na}} = \frac{c}{M_n^{eq}} + \tfrac{1}{2}\sum_i \sum_j B_{ij} c_i^o c_j^o \quad (15)$$

Here

$$\frac{c}{M_n^{eq}} = \frac{c_A}{M_A} + \frac{c_B}{M_B} + \frac{K c_A^n c_B^m}{M_{A_nB_m}} \quad (16)$$

Fig. 2A shows a plot of $1/M_{na}$ vs. c at 37 °C for an ovalbumin (A) and lysozyme (B) blend having $\beta = 1.014$. It is evident from this plot that there is no mixed association (see eq. 11 and the preceding paragraph). The values of B_{AA} and B_{BB} are the osmotic pressure second virial coefficients; they are obtained from measurements at the same temperature on solutions containing only ovalbumin (A) or lysozyme (B). Knowing B_{OS}, B_{AA} and B_{BB}, eq. 12 is used to obtain B_{AB}. Fig. 2B shows a plot of $1/M_{na}$ vs. c at 37 °C and $\beta = 0.532$ and fig. 2C gives the corresponding plot at 30 °C and $\beta = 1.012$. The values of M_n are recorded in table 1, and those of B_{OS} are listed in table 2. Table 3 lists the values of B_{AB} obtained from these measurements. The expected values of M_n^o are calculated from

$$\frac{1}{M_n^o} = \frac{\beta}{M_A(1+\beta)} + \frac{1}{M_B(1+\beta)} \quad (17)$$

and are also recorded in table 1. These values are

Table 3

Results for B_{AB} in experiments 5–7, with standard errors

Concentration ratio $\beta = c_A^o/c_B^o$	B_{AB} (1 mol g^{-2}) ($\times 10^6$)	
	$T = 310$ K	$T = 303$ K
1.014	-2.0 ± 0.3 [a]	–
	-2.0 ± 0.3 [b]	–
0.532	-1.4 ± 0.3 [a]	–
	-1.4 ± 0.3 [b]	–
1.012	–	-2.0 ± 0.4 [a]
		-1.6 ± 0.2 [b]

[a] Values calculated from eq. 12.

[b] Values computed from slope of tabulated ($1/M_{na}^*$) vs. c data.

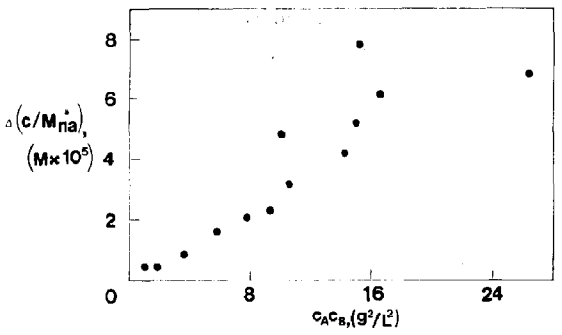

Fig. 3. Plots of $\Delta(c/M_{na}^*)$ vs. c_Ac_B based on eq. 19 for the ovalbumin-lysozyme blend at 37°C having $\beta = 0.532$. Although there is a lot of scatter in the plot, we were able to fit this plot with a straight line; the slope of this line gives K/M_{AB}. Similar results were obtained with the two other blends. The values of K, the association constant, are listed in table 4.

indicated by an × on the ordinates in fig. 2A–C. The differences in the expected and actual intercepts are −3.7, −0.9 and +2.5% for fig. 2A–C, respectively. These differences are less than or equal to the sum of the relative standard errors of the expected and extrapolated intercepts.

It is also possible to obtain B_{AB} another way from these measurements. A quantity M_{na}^* is defined as follows:

$$\frac{1}{M_{na}^*} = \frac{1}{M_{na}} - \left[\frac{\beta B_{AA}}{(1+\beta)^2} + \frac{B_{BB}}{(1+\beta)^2}\right]\frac{c}{2}$$

$$= \frac{1}{M_n} + \beta B_{AB} c/(1+\beta)^2 \qquad (18)$$

Thus, at constant β with no mixed association present, a plot of $1/M_{na}^*$ vs. c has an intercept of $1/M_n$ and a slope of $\beta B_{AB}/(1+\beta)^2$. We have shown such plots for each of the three solutions in fig. 2A–C; these plots have a dashed line. The values of B_{AB} obtained from eq. 18 are listed in table 3.

We attempted to analyze the data as a quasi-ideal mixed association. Suppose that one assumes that $B_{AB} = 0$ and that the nonideal effects were due to B_{AA} and B_{BB} only, then from eq. 11 one

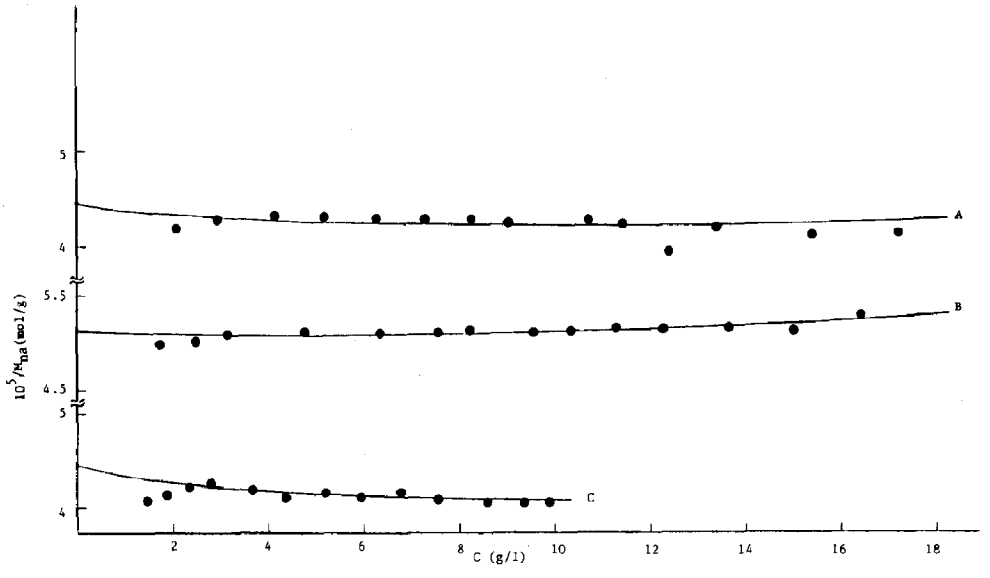

Fig. 4. Plots of $1/M_{na}$ vs. c for the three ovalbumin-lysozyme blends: (A) 37°C, $\beta = 1.014$; (B) 37°C, $\beta = 0.532$ (C) 30°C, $\beta = 1.012$. The solid line is based on the regenerated data obtained using the appropriate values of K, B_{AA}, B_{BB} and c for each blend. Note how well the regenerated plots describe the experimental points (●). This indicates that these data can be described by an A + B ⇄ AB mixed association as well.

notes that

$$\Delta(c/M_{na}^*) = (K/M_{AB})c_A c_B$$
$$= (K/M_{AB})[c_A^\circ - \Delta(c/M_{na}^*)M_A]$$
$$\times [c_B^\circ - \Delta(c/M_{na}^*)M_B] \quad (19)$$

Fig. 3 shows a plot of $\Delta(c/M_{na}^*)$ vs. $c_A c_B$ for experiments performed at 37 °C and $\beta = 0.532$. Although there is a lot of scatter in the data, we were able to fit a straight line through the points (two points which were considerably off the straight line were omitted in these calculations); here we performed regression analysis on an equation of the form $y = mx$ [38]. For this plot we obtained 0.323×10^{-5} for the slope. Assuming $M_{AB} = 5.84 \times 10^4$, then $K = 0.189$ l/g. This can be converted to $k = 2.12 \times 10^3$ l/mol. Similar plots based on eq. 19 were made for the other two experiments; these plots also showed a lot of scatter. Table 3 shows the values of K and k obtained in these experiments. Note that at the same temperature but at different values of β, the value of K differed considerably; this may be due to experimental error. When the $1/M_{na}$ vs. c data were regenerated using the experimentally determined values of K, B_{AA} and B_{BB}, it was found that the regenerated data gave a very good description of the original data. This is shown in fig. 4; it is evident from the regenerated data (solid lines) that the mixed association is a weak one. Thus, the assumption that a quasi-ideal mixed association was present was quite reasonable, and it places our results in accord with previous studies. The scatter in the plots in fig. 3 may be due to the fact that here one has a relatively weak mixed association present, and in order to evaluate K, one must obtain $\Delta(c/M_{na}^*)$. Here, it means that one is taking small differences, which, because of the experimental error, results in the scatter. Had the association been stronger, then the scatter might have been less, and also the plot of $1/M_{na}^*$ vs. c would show the curvature that one would expect with a mixed association.

4. Discussion

First, we will discuss some observations on the experimental technique. The 298.2 K (25 °C) temperature of the gel-filtration experiments [17] could not be attained with the Knauer membrane osmometer. According to the manufacturer, the temperature control of the osmometer works from 5 °C above ambient temperature to 120 °C. At 310.2 K (37 °C) temperature equilibrium was rapidly attained and maintained. Temperature instability may have added to the scatter in the 30 °C data. It was critical to remove bubbles from the tubing connecting the calibration device to the osmometer. Pressure baselines were stable to ± 0.01 cm H_2O; there was some infrequent drift due to the chart recorder. Before beginning the measurements one should always check the chart recorder zero. During calibration one may encounter a very slight positive slope in the 10 cm tracing. This problem can be minimized by making sure that the tubing fits tightly to the fittings on the osmometer. The average deviation in the full-scale deflection was usually $\pm 0.1\%$. It is important to degas not only all solutions used in membrane osmometry but also the membrane, using a vacuum desiccator connected to a vacuum line or to an aspirator. During the degassing of protein solutions some foaming was observed at the meniscus, but the foam dissipated upon removal of the vacuum. Although any membrane used in dialysis could be used in membrane osmometry, we have had best results with cellulose acetate membranes, such as the Schleicher and Schuell AC61 or AC62 membranes. Some dialysis membranes are very retentive and are slower in attaining equilibrium. A new asymmetric, thin film membrane (available from Wescan Instruments), which can attain osmotic equilibrium in 5 min for molecules having a molecular weight of 15000 g/mol or more, has been announced recently.

As ovalbumin solutions were mixed with lysozyme solutions, turbidity developed immediately; this was followed by a slower precipitation. After filtration the solutions remained clear indefinitely. In order to quantify the material loss, the refractive index difference, Δn, was measured after filtration and compared to the expected value of Δn (assuming no volume change on mixing) in the mixture. The change in Δn in one experiment was -3.2×10^{-5}, which corresponds to a protein loss of about 0.8%. Thus, no corrections were made to

the stock concentrations. Precipitation from ovalbumin-lysozyme mixtures has been reported previously [19]. Similarly, turbidity has been encountered in mixtures of bovine serum albumin and lysozyme; this turbidity could be reduced on increasing the ionic strength [30]. We did not pursue this effect of ionic strength, since the ovalbumin-lysozyme mixed association is thought to occur only at low ionic strengths.

At low concentrations of ovalbumin values of Π, the osmotic pressure, were somewhat lower than would be expected based on the Π/c vs. c plot. This could be due to adsorption of the protein at low concentrations; it might also be due to a nonlinear response of the pressure transducer at lower pressures. The pressure reduction effect was less pronounced in lysozyme or in ovalbumin-lysozyme solutions. Nonetheless, the molecular weights obtained for ovalbumin or for lysozyme at the two temperatures are consistent with each other and are in agreement with published values for these proteins.

The average result for the molecular weight of ovalbumin was $(4.295 \pm 0.04) \times 10^4$ g/mol. This compares favorably with values obtained from sedimentation equilibrium experiments: 43 500 [31] and 43 040 [25] g/mol. The osmotic pressure value for M is less than the value of 45 700 g/mol obtained by Halwer et al. [22] using light scattering, or the value 44 620 g/mol obtained by Castellino and Barker [25] by membrane osmometry. Had we used the value of 0.182 ml/g for the refractive index increment of ovalbumin at $\lambda = 546$ nm [22], we would have obtained $(4.437 \pm 0.04) \times 10^{-4}$ g/mol as the molecular weight.

For lysozyme we obtained molecular weight values of $(15.1 \pm 0.45) \times 10^3$ at 37 °C and $(16.0 \pm 0.4) \times 10^3$ g/mol at 30 °C. At 37 °C the lower limit for M is 14.65×10^3 g/mol; this is in accord with the value $M = 14\,307$ g/mol obtained by amino acid sequencing [32]. It is also in accord with the value $M = 14\,400 \pm 100$ g/mol obtained from sedimentation equilibrium experiments [26]. The value of M at 30 °C appears to be significantly higher; this might be due to a weak self-association or it might be a consequence of poorer temperature control at 30 °C. Since self-association of lysozyme solutions was found at pH 5.8 in 0.15 M KCl at 293.2 K (20 °C) [33], it would not be surprising if self-association did occur at 0.02 ionic strength and pH 5.8 at 30 °C. Due to the absence of good data at low concentrations, the very small dimerization constant (if a self-association is present) could not be evaluated here. However, the osmotic pressure data can be represented with adequate precision using the higher M value along with a second virial coefficient. The value of the refractive index increment, ψ, used influenced the value of M obtained; if we had used another value for ψ, namely, 0.185 ml/g, which was used at pH 7.0 in 0.2 ionic strength cacodylate buffer [34], then the value for M would have been $(15.43 \pm 0.45) \times 10^3$ g/mol at 37 °C and $(16.35 \pm 0.40) \times 10^3$ g/mol at 30 °C.

The osmotic pressure second virial coefficients showed no significant temperature dependence in these experiments (see table 2). The values of B_{AA} and B_{BB} are greater than expected for native proteins due to excluded volume alone. Castellino and Barker [25] determined B_{OS} for ovalbumin at pH 6.0 in 0.1 M potassium phosphate buffer containing 0.1 M NaCl. Their value of $(2.40 \pm 0.17) \times 10^{-8}$ l mol g^{-2} corresponds to $(4.8 \pm 0.3) \times 10^{-8}$ l mol g^{-2} by our definition (see eq. 2). The value of B_{AA} in this work is about 12-times larger, even though ovalbumin is less charged at pH 5.8. Excluded volume contributions to B are generally proportional to the specific volume of the protein [35], and ovalbumin is noted to gain an appreciable charge without a change in its specific volume (see ref. 35, p. 516). Since the ionic strength is small, it is reasonable to attribute some of the magnitudes of B_{AA} and B_{BB} to the Donnan effect. In a three-component system (water, uni-univalent electrolyte, protein) the ideal Donnan contribution B_D to the second virial coefficient is [36]

$$B_D = \frac{M_3 z^2}{4 c_3 M_2^2} \tag{20}$$

where c_3/M_3 is the molar concentration of the uni-univalent electrolyte, M_2 the protein molecular weight and z the charge on the protein ion. If one sets $B_D = B_{AA}$, $M_2 = 43\,000$ and $c_3/M_3 = 0.02$ one obtains $z^2 = 81.4$. This predicts $z_A = -9.0$. An estimation of the charge of ovalbumin based

on titration data at ionic strength $I = 0.017$ gives the charge on ovalbumin at pH 5.8 as -7.0 [37]. Thus, the evidence is compelling that B_{AA} in this work is largely due to the Donnan effect. For lysozyme the charge estimated from titration data at $I = 0.03$ and $T = 298$ K is $+8$ [38]. The ideal Donnan contribution for lysozyme is expected to be $B_D = 3.6 \times 10^{-6}$. The average value of B_{BB} in this work is less than B_D by a factor of 0.43, which may be reasonable since self-association or other factors may affect B_{BB} in the opposite direction to the Donnan effect.

It is evident from a comparison of fig. 2A and B that the value of β influence B_{OS}. Recalling that $\beta = c_A^o/c_B^o$ and that A represents ovalbumin and B lysozyme, one notes that at 37 °C (310.2 K) if the blend is richer in ovalbumin ($\beta = 1.014$) then B_{OS} is negative, whereas at $\beta = 0.532$ where the blend is richer in lysozyme, then the slope is slightly positive. At 30 °C B_{OS} is negative when the mixture is richer in ovalbumin ($\beta = 1.012$). At similar β values (1.014 and 1.012) we obtained virtually the same values for B_{OS}. In both cases we obtained virtually the same value for B_{AB}. Furthermore, the value for B_{AB} at 310.2 K (37 °C) and $\beta = 0.532$ was almost the same, within experimental error, as that for B_{AB} at $\beta = 1.014$. It is interesting to note that we could not detect any effect of temperature on the nonideal terms, but that we were able to detect the mixed nonideal term, B_{AB}, which indicates that each solute affects the activity of the other.

At constant β one would expect that the plot of $1/M_{na}$ (or the plot of the osmotic coefficient, $g = M_n^\circ/M_{na}$) vs. c for a mixed association would show some curvature, such as was encountered in previous studies on the mixed association between cholesterol and methyl cholate in $CHCl_3$ solutions at 37 °C (see fig. 3 of ref. 10). The shape of the curve would depend on the values of the values of the equilibrium constant(s) and the nonideal terms. Thus, we were quite surprised to find that our data could be described as a quasi-ideal mixed association. The plot based on eq. 19 shows a lot of scatter (see fig. 3); this may be due to the fact that one is taking small differences in the determination of $\Delta(c/M_{na}^*)$. In the plots shown in fig. 4 one is not taking small differences, but here one plots the data directly. Experimental error will cause more scatter if the equilibrium constant is small, as is the case here. With stronger association constants, one should encounter less scatter in the plots based on eq. 19. This could also be responsible for the difference in K values at the same temperature; however, in previous vapor osmometric studies on the mixed association between cholesterol and methyl cholate in $CHCl_3$ solutions at 37 °C, Foster et al. [10] also encountered variability in the values of the association constant.

Nichol et al. [17] also studied the mixed association of ovalbumin and lysozyme at 25 °C in 0.06 M ($I = 0.02$, pH = 5.8) cacodylate buffer using gel-filtration chromatography on Sephadex G-100 columns. In order to perform their calculations they assumed ideal solution conditions and had to make some additional assumptions in order to evaluate some of the constituent concentrations of the reactants. Their equilibrium constants were based on constituent concentrations of the various interacting species. The concentrations used covered the ranges $(1.12–1.55) \times 10^{-4}$ M or 5.0–7.0 g/l for ovalbumin and $(2.01–2.10) \times 10^{-4}$ M or 2.9–3.1 g/l for lysozyme. The mean association constant was 5.2×10^4 M^{-1} with a range of $(4.2–5.8) \times 10^4$ M^{-1}. These values are an order of magnitude greater than those we encountered (see table 4).

Studies on the mixed association of ovalbumin and lysozyme at 25 °C have been carried out in phosphate buffer at pH 6.80 and in phosphate buffer of ionic strength 0.02. By gel-filtration

Table 4

Equilibrium constants for the A + B + ⇄ AB association of ovalbumin and lysozyme in 0.06 M cacodylic acid buffer ($I = 0.02$, pH = 5.8)

t (°C)	β [a]	K [b] (l/g)	K ($\times 10^{-3}$) (M^{-1})
37	0.532	0.189 ± 0.004	2.12 ± 0.04
37	1.014	0.379 ± 0.013	4.45 ± 0.15
30	1.012	0.211 ± 0.009	2.37 ± 0.10

[a] $\beta = c_A^o/c_B^o$

[b] Obtained by least squares [39].

chromatography Nichol and Winzor [15] interpreted their data as an $A + B \rightleftarrows C$ association with an apparent association constant ranging from $(2.0–3.2) \times 10^4$. Sedimentation equilibrium studies performed under the same conditions by Howlett and Nichol [18] indicated the presence of aggregates larger than AB. Additional gel-filtration and sedimentation equilibrium experiments led Jeffrey et al. [19] to conclude that there is an array of aggregates involving cross-linked and/or noncross-linked aggregates. Obviously, the interpretation of the mixed association data becomes more complicated at low ionic strength with pH values greater than 5.8. Our study at pH 5.8 did not indicate the presence of aggregates greater than the AB complex.

In vapor pressure osmometric studies of quaternary ammonium tetrahaloferrates in organic solvents, Kertes and Markovits [40] analyzed their data in two ways: first, they calculated activity coefficients and osmotic coefficients via Gibbs-Duhem relationships, and second, they also analyzed the data as a self-association. At constant β, similar considerations should apply to mixed associations; thus, it is not surprising that there are two different interpretations of the data presented here.

Our results and those of Nichol et al. [17] indicate that a mixed association is present between ovalbumin and lysozyme. In the fertilized chicken egg lysozyme would protect the embryo from bacterial infection by dissolving the bacterial cell walls. The rectal temperature of a chicken ranges from 40.6 to 43.0 °C [41], so it would seem that at those temperatures the lysozyme should be uncomplexed or very weakly complexed (as is the case here) so that it is available for antibacterial activity. It is evident from these studies that one can indeed study mixed associations. The theory for analyzing mixed associations by osmometric methods is much simpler than that for elastic light scattering or for sedimentation equilibrium experiments. In these experiments we were able to detect nonideal behavior of the reactants and include these nonideal terms in our analysis. In the gel-filtration analysis Nichol et al. [17] had to assume completely ideal solution conditions, and they were required to make assumptions regarding the elution volumes from the elution profile of a boundary in order to analyze their data.

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