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STUDIES OF MACROMOLECULAR HETEROGENEOUS ASSOCIATIONS INVOLVING CROSS-LINKING: A RE-EXAMINATION OF THE OVALBUMIN–LYSOZYME SYSTEM

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A system is considered in which a multivalent acceptor interacts with a bivalent ligand in solution to form an array of complexes via multiple binding and cross-linking reactions. With the use of reacted site probability functions expressions are derived in terms of a site binding constant which are of potential use in the interpretation of sedimentation equilibrium and binding results obtained with such systems. Their potential use is explored in relation to results obtained on the interacting ovalbumin–lysozyme system at pH 6.80, ionic strength 0.02. A comparison is made of this interpretation with that based on an interaction pattern involving only multiple binding of ligand in the absence of cross-linking effects. While both interpretations quantitatively describe certain results, it is shown, by invoking further experimental observations on apparent weight-average molecular weight and precipitation behavior, that the more favored interpretation is that involving the operation of a spectrum of forces leading to a large array of ovalbumin–lysozyme complexes, including those of the cross-linked type. It is stressed that the particular ovalbumin–lysozyme system is but one example of interaction between oppositely charged macromolecules and therefore that the derived equations may find wider application to such systems and those known to involve more specific cross-linking interactions.

1. Introduction

Interactions between a multivalent acceptor and a bivalent ligand leading to an array of complexes comprising alternating acceptor and ligand molecules have been studied in relation to the formation of synthetic non-linear polymers [1–3] and have formed the basis for elucidating certain antigen-antibody systems [4–6]. Moreover, it has been suggested that such cross-linking reactions may be of relevance in the heterogeneous association of other types of protein [7]. Recently, a binding theory has been developed [7] in terms of reacted site probability functions [2,8] which offers considerable potential in the study of cross-linking reactions. Its use to date has been limited to an analysis of radioimmunoassay results obtained with sperm whale myoglobin and its specific antibodies [7] and it is noted that this experimental approach is both specialized and quantitatively imprecise. More general experimental methods and analysis procedures are needed to explore the possibility that other macromolecular heterogeneous associations are of the cross-linking type. Two experimental methods are likely to be particularly useful in relation to the interaction theory based on reacted site probability functions. First, a frontal chromatographic procedure has been devised [9] which yields for macromolecular systems, the relationship between the moles of ligand bound per mole of total acceptor and the molar concentration of unbound ligand: this is precisely the information required for the direct application of the general binding equation for cross-linking systems [7]. Secondly, it has been shown that the method of sedimentation equilibrium may yield the molar concentrations of unbound acceptor and ligand as a function of total concentration [10]. It is one purpose of this work to show that such information, itself independent of the reaction pattern, permits evaluation of reacted site probability functions and hence leads to a method for testing and elucidating cross-linking interactions. It is noted that theory pertaining to the sedi-

mentation equilibrium of heterogeneously associating systems has hitherto been restricted to the consideration of systems in which a discrete number of complexes is formed [10,11–14], whereas this work considers for the first time an indefinite heterogeneous association involving an extensive array of complexes.

Although the theory to be presented is of general applicability to systems of the cross-linking type, it was felt desirable to illustrate its use and to explore its quantitative potential with experimental results obtained with a model system. The interaction between ovalbumin and lysozyme at pH 6.8 in phosphate buffer of ionic strength 0.02 was selected and consideration is given to results obtained on this system previously [9,14] and in this work. Two possible reaction patterns are considered in parallel. The first envisages a simple multiple binding of lysozyme (B) to ovalbumin (A) leading to the formation of a limited number of complexes of the type AB_j. The second acknowledges the distinct possibility that interactions between oppositely charged macromolecules in solution may not be so limited, but rather may lead to an extensive array of complexes of the type A_iB_j via a mixture of multiple binding and cross-linking effects.

2. Theory

2.1. Basic relations for the cross-linking system

Consider first the interaction between a multivalent acceptor, A, possessing f equivalent and independent reactive sites, and a bivalent ligand, B. At equilibrium in solution, there will coexist unbound B and a series of complexes A_iB_j ($i = 1,2,...,\infty$; $j = (i-1), i, (i+1), ..., i(f-1)+1$), where $i = 1, j = 0$ denotes unbound A. This array of complexes has been delinated in eq. (1) of ref. [7]. The reacted site probability function, P_A, is defined as the probability that any given site on an A molecule has reacted with a given site on a ligand molecule, such that the total molar concentration of the acceptor in the mixture, $\overline{m}_A$, is related to its equilibrium unbound concentration, m_A, by

$$\overline{m}_A = m_A/(1-P_A)^f; \qquad 0 \leqslant P_A < 1. \tag{1}$$

With similar notation, we may write for the bivalent ligand species

$$\overline{m}_B = m_B/(1-P_B)^2; \qquad 0 \leqslant P_B < 1. \tag{2}$$

A site binding constant, k, is now defined as the ratio of the concentration of reacted A sites ($fP_A\overline{m}_A$) to the product of the concentration of unreacted A sites ($f(1-P_A)\overline{m}_A$) and B sites ($2(1-P_B)\overline{m}_B$), so that

$$k = P_A/2(1-P_A)(1-P_B)\overline{m}_B. \tag{3}$$

Since $fP_A\overline{m}_A = 2P_B\overline{m}_B$, it follows that eq. (3) may be rewritten as

$$k = P_B/f(1-P_A)(1-P_B)\overline{m}_A. \tag{4}$$

2.2. Sedimentation equilibrium

At sedimentation equilibrium eqs. (1)–(4) apply at each radial position, x, and may be combined in the following way to permit evaluation of $P_A(x)$ and $P_B(x)$. Multiplication of eqs. (1) and (2), and of eqs. (3) and (4) and combination of the resulting expressions yields

$$k^2 = P_A(x)P_B(x)/2f(1-P_A(x))^{2-f}m_A(x)m_B(x). \tag{5}$$

Moreover, combination of eqs. (2) and (3) gives

$$k = P_A(x)(1-P_B(x))/2(1-P_A(x))m_B(x) \tag{6}$$

or

$$P_B(x) = 1 - \{2k(1-P_A(x))m_B(x)/P_A(x)\}. \tag{7}$$

Division of eq. (5) by eq. (6) and substitution for $P_B(x)$ from eq. (7) yields [7],

$$2fk^2m_A(x)m_B(x)(1-P_A(x))^{2-f} - P_A(x)(1+2km_B(x)) + 2km_B(x) = 0. \tag{8}$$

Eqs. (7) and (8) provide a useful basis for analysing sedimentation equilibrium results obtained at constant angular velocity, ω, and temperature, T, since values of $m_A(x)$ and $m_B(x)$ may be deduced in the following way [10]. It is possible to select, from an experimental distribution of the total weight concentration $\overline{c}(x)$ versus x, a reference point, $[\overline{c}(x_F), x_F]$, and to calculate values of the function $\Omega_B(x)$ where B refers to the reactant species with the lower product of molecular weight, M, and buoyancy term, $(1-\overline{v}\rho)$

$$\Omega_B(x) = \overline{c}(x)\exp\{\phi_B M_B(x_F^2 - x^2)\}/\overline{c}(x_F), \tag{9a}$$

$$\phi_B = (1 - \bar{v}_B \rho)\omega^2/2RT. \quad (9b)$$

A plot of $\Omega_B(x)$ versus $\bar{c}(x)$ yields on extrapolation $\Omega_B^0(x)$ which has been shown [10] to equal $a_B(x_F)/\bar{c}(x_F)$ where $a_B(x_F)$ is the thermodynamic activity of unbound B at the reference position. It follows that

$$m_B(x) = \bar{c}(x)\Omega_B^0(x)/M_B\Omega_B(x) \quad (10)$$

on the assumption that thermodynamic non-ideality effects are sufficiently small to permit identification of $a_B(x)$ and $c_B(x) = M_B m_B(x)$. In the second step, values of $\bar{c}^*(x)$ are determined as the difference, $\bar{c}(x) - c_B(x)$, and used in an analogous procedure to find for the A reactant,

$$\Omega_A(x) = \bar{c}^*(x)\exp\{\phi_A M_A(x_F^2 - x^2)\}/\bar{c}^*(x_F), \quad (11a)$$

$$m_A(x) = \bar{c}^*(x)\Omega_A^0(x)/M_A\Omega_A(x). \quad (11b)$$

The values of $m_A(x)$ and $m_B(x)$, thus determined, are independent of the nature of the interaction pattern.

Consider now any particular point on the experimentally obtained sedimentation equilibrium distribution where a value of $\bar{c}(x)$ is known and given by

$$\bar{c}(x) = M_A\bar{m}_A(x) + M_B\bar{m}_B(x). \quad (12)$$

For a selected integral value of $f \geqslant 2$, it is possible to utilize the determined values of $m_A(x)$ and $m_B(x)$ at the chosen point to solve numerically eqs. (8), (7), (1) and (2), respectively, for physically permissible values of $P_A(x)$, $P_B(x)$, $\bar{m}_A(x)$ and $\bar{m}_B(x)$ for a range of values of k and hence to determine the value of k appropriate to the selected point. Repetition of this procedure for other values of f leads to a series of corresponding values of f and k which define the selected $[\bar{c}(x),x]$ point in terms of the cross-linking model. A test of the relevance of this model and the potential delineation of the appropriate values of f and k may proceed by fitting the entire sedimentation equilibrium distribution, and indeed those obtained in other experiments conducted with solutions of different initial compositions, with the use of eqs. (1), (2), (7) and (8). Thus, direct comparisons are possible between $\bar{c}(x)$ values determined experimentally and calculated in this way.

Evaluation of $m_A(x)$ and $m_B(x)$ from a sedimentation equilibrium distribution is also useful in examining the possible applicability of a multiple binding pattern of the type, $AB_{j-1} + B \rightleftharpoons AB_j$ $(j = 1,2,...,f)$ involving no cross-linking of acceptor molecules. For such a system where the f sites on A are equivalent and independent so that a single intrinsic binding constant, k_A, suffices to describe all equilibria, it has been shown [15,16] that

$$\bar{m}_A(x) = m_A(x)(1 + k_A m_B(x))^f, \quad (13a)$$

$$\bar{m}_B(x) = m_B(x) + fkm_A(x)m_B(x)(1 + k_A m_B(x))^{f-1}. \quad (13b)$$

Clearly, use of eqs. (13a) and (13b) together with eq. (12) permits sets of values of f and k_A to be evaluated which are consistent with a particular point on a sedimentation equilibrium distribution where the values of $m_A(x)$ and $m_B(x)$ have been evaluated. The relevance of these values may then be tested in relation to the entire distribution (or other distributions), using the same set of equations.

2.3. Binding experiments

In relation to the reaction pattern $AB_{j-1} + B \rightleftharpoons AB_j$, the binding function r $(= (\bar{m}_B - m_B)/\bar{m}_A)$ may be formulated from eqs. (13a) and (13b) as

$$r = fk_A m_B/(1 + k_A m_B). \quad (14)$$

For the cross-linking pattern involving complexes of the type $A_i B_j$, it has been shown [7] that the same binding function r may be written in terms of $\bar{m}_A$ and m_B as follows

$$r = m_B\{[1/(1 - P_B)^2] - 1\}/\bar{m}_A, \quad (15a)$$

or

$$r = \{2fkm_B/(1 + 2km_B)\} + \psi, \quad (15b)$$

$$\psi = \frac{(1 - 4k^2m_B^2)}{8k^2\bar{m}_A m_B} \times \left\{1 + \frac{4fk^2\bar{m}_A m_B}{(1+2km_B)^2} - \left[1 + \frac{8fk^2\bar{m}_A m_B}{(1+2km_B)^2}\right]^{1/2}\right\}. \quad (15c)$$

It has already been noted that a particular design of frontal chromatography [9] is an appropriate method for determining values of r and m_B at known values of $\bar{m}_A$ for interacting macromolecular systems.

It is clear then that eqs. (14) and (15) may be used to examine such experimental results in terms of both the multiple binding and cross-linking models: in particular, they allow values of f and k_A or k (as appropriate) indicated from the analysis of sedimentation equilibrium results to be correlated with binding results. Eq. (15) has not hitherto been used to examine experimental results, although an alternative form has been employed to curve-fit radioimmunoassay data when the percentage of A bound was known [7].

3. Experimental

Five-times crystallized ovalbumin (Sigma) and twice-crystallized hens' egg white lysozyme (Worthington) were used without further purification. Stock solutions were prepared by dissolving the proteins separately in phosphate buffer (0.005 M NaH_2PO_4, 0.005 M Na_2HPO_4), pH 6.80, ionic strength 0.02, and concentrations of dialysed solutions were determined spectrophotometrically employing the values of 6.6 and 27.2 for the extinction coefficients ($E^{1\%}_{1\,cm}$) at 280 nm of ovalbumin [17] and lysozyme [18], respectively. Mixed solutions were prepared by weight using the pre-dialysed stock solutions filtered through a 0.22 μm millipore membrane: checks were made that the pH of the mixed solutions remained at the value of 6.80. In general, a turbidity developed on mixing stock solutions and, in the construction of a precipitin curve to investigate the effect, the extent of this was estimated by turbidimetry at 360 nm immediately after mixing and prior to the onset of any flocculation. Other experiments on such mixed solutions were performed after filtration and re-dialysis using Visking 8/32 tubing. It was found in control experiments that the amount of solid material thus removed never exceeded 2% of the total macromolecular solute and that the filtered solutions remained clear: initial compositions of mixtures to be reported are accordingly defined in terms of the proportions and concentrations of the stock solutions as mixed.

Sedimentation equilibrium experiments were performed in a Spinco model E ultracentrifuge equipped with electronic speed control and interference optics. The procedure for the conduct of these experiments (performed at 25 ± 0.1°) and for the measurement of Rayleigh interference patterns has been described previously [14,19,20]. In the analysis of these results the following values were assigned to the required parameters: the specific refractive increments of all macromolecular species were taken as 0.00185 dl/g [21]; the partial specific volumes of all species were also taken to be identical with a value of 0.73 ml/g [22]; the molecular weights used for the acceptor A (ovalbumin) and ligand B (lysozyme) were 45 000 [23] and 14 400 [22], respectively. Comment is now required on any possible self-association of the reactants. There is considerable evidence summarized in ref. [23] that ovalbumin does not self-interact near neutrality. On the other hand, lysozyme is known to self-associate weakly at pH 6.7 and ionic strength 0.17 [19,24]. Accordingly, a control sedimentation equilibrium experiment was performed on lysozyme alone at pH 6.80 and ionic strength 0.02, values relevant to this work. The results indicated, in accordance with the concept of increased electrostatic repulsion at the lower ionic strength, that the self-association of lysozyme had become even weaker. A maximum value of 0.2 dl/g could be assigned to the apparent dimerization constant, indicating, for example, that a solution of concentration of 1 g/liter would contain at the most, only 2% of the total solute as dimer. This was neglected in the analysis of results on mixed solutions where, as we will see, effects of heterogeneous association dominate the experimental behavior.

Binding experiments conducted to evaluate r as a function of m_B at fixed $\bar{m}_A$ were performed using a Sephadex G-75 (1.6 X 44 cm) column, thermostatted at 25° and equilibrated with the pH 6.8 phosphate buffer. The pre-filtered mixed solutions were applied with loading volumes of approximately 80 ml, sufficient to ensure a plateau region of initial solution composition in the elution profiles. These were obtained by eluting with the phosphate buffer at a constant flow rate of 14 ml/h and by monitoring tared fractions spectrophotometrically. As described previously [9], the trailing plateau in each profile was comprised of lysozyme alone at a concentration equal to its equilibrium value, m_B, in the plateau of original composition defined by known values of $\bar{m}_A$ and $\bar{m}_B$, since unbound ovalbumin and all complexes comigrated at the void volume.

4. Results and discussion

Two previous studies have been performed on the interaction between ovalbumin and lysozyme in the solution environment used in this work. In the first [9], binding results were interpreted with some reservation in terms of the formation of a single 1 : 1 complex of molecular weight 59 400. In the second more recent study [14], this interpretation was shown to be incorrect since apparent weight-average molecular weights as high as 100 000 were found by sedimentation equilibrium. The latter authors, while favoring a reaction pattern in which the A_2B_2 complex predominated at relatively high total protein concentration, did not claim that a pattern had been found which fitted their results within available experimental precision. Indeed, these authors referred to a scheme involving the complexes AB, AB_2, A_2B, A_2B_2 and higher complexes, but had no tractable means of analysing their results in terms of such an array of complexes. Nevertheless, the existence of an array of complexes is not unreasonable on physical grounds since at pH 6.8 the net charges borne by ovalbumin and lysozyme are approximately −14 and +7.5, respectively [23,25], and a variety of electrostatic interactions are likely to contribute significantly to the total free energy of the system at the low ionic strength (0.02) employed.

Two sedimentation equilibrium experiments were considered in this work in relation to complex formation between ovalbumin and lysozyme via either a multiple binding process (leading to the complexes AB_j) or interactions involving cross-linking (to form the array of complexes A_iB_j). The first was conducted with a solution of initial composition, $\overline{m}_A = 0.798 \times 10^{-5}$ M and $\overline{m}_B = 1.683 \times 10^{-5}$ M, at an angular velocity of 20 000 rev/min and the second, performed previously [14], utilized $\overline{m}_A = 6 \times 10^{-5}$ M, $\overline{m}_B = 9.7 \times 10^{-5}$ M and $\omega = 7811$ rev/min. The first two columns of table 1 summarize experimentally obtained $(x, \bar{c}(x))$ points describing these sedimentation equilibrium distributions: for brevity, only six points

Table 1
Analysis of sedimentation equilibrium results obtained with the ovalbumin–lysozyme system in phosphate buffer of pH 6.80 at 25°C

Experimental data				Cross-linking model $f = 4, k = 0.14 \times 10^4$ M^{-1}				Multiple-binding model $f = 4, k_A = 0.35 \times 10^4$ M^{-1}	
x (cm)	$\bar{c}(x)$ (g/liter)	$m_A(x)$ ($\times 10^5$ M)	$m_B(x)$ ($\times 10^5$ M)	$P_A(x)$	$P_B(x)$	$\bar{c}_{calc}$ (g/liter)	Δ (g/liter)	$\bar{c}_{calc}$ (g/liter)	Δ (g/liter)
Expt. 1.									
6.8675	0.141	0.054	0.697	0.020	0.003	0.128	0.013	0.129	0.012
6.9223	0.182	0.116	0.909	0.026	0.007	0.192	0.010	0.193	0.011
6.9770	0.303	0.249	1.188	0.033	0.016	0.306	0.003	0.311	0.008
7.0318	0.521	0.538	1.556	0.044	0.034	0.532	0.011	0.545	0.024
7.0865	1.002	1.167	2.041	0.059	0.075	1.015	0.013	1.047	0.045
7.1230	1.680	1.964	2.449	0.074	0.124	1.665	0.015	1.708	0.028
							SD = ± 0.013		± 0.027
Expt. 2.									
6.8784	2.928	1.983	5.497	0.158	0.159	2.903	0.025	2.976	0.048
6.8925	3.053	2.048	5.559	0.160	0.165	3.007	0.040	3.077	0.024
6.9235	3.295	2.196	5.683	0.165	0.177	3.251	0.044	3.302	0.007
6.9518	3.545	2.342	5.800	0.170	0.190	3.502	0.043	3.528	0.017
6.9781	3.793	2.487	5.917	0.175	0.202	3.764	0.029	3.759	0.034
7.0011	4.038	2.622	6.014	0.179	0.214	4.008	0.030	3.973	0.065
							SD = ± 0.039		± 0.040

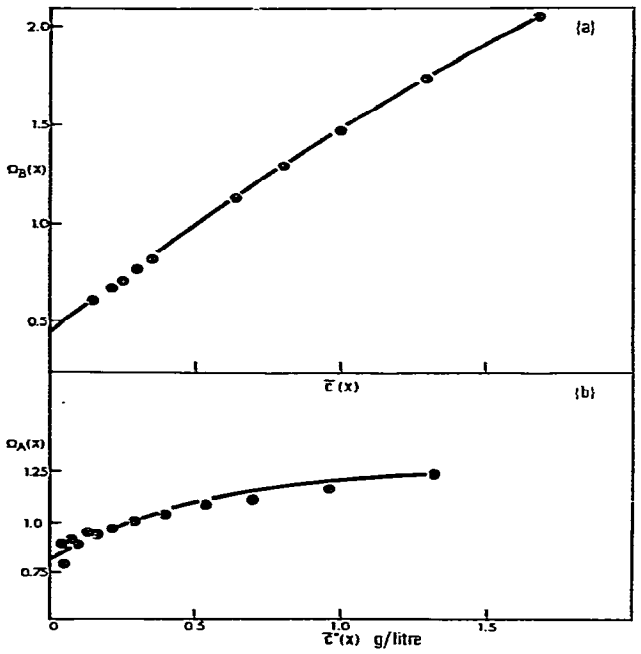

Fig. 1. Sedimentation equilibrium results obtained after 20 h at 20 000 rev/min and 25°C with a solution of initial composition 0.798×10^{-5} M ovalbumin and 1.683×10^{-5} M lysozyme. The column height was 0.27 cm. (a) A plot of $\Omega_B(x)$ as defined by eq. (9a) versus the total solute concentration $\bar{c}(x)$. The reference point selected was $x_F = 7.0318$ cm and $\bar{c}(x_F) = 0.521$ g/liter. The solid line shows the extrapolation of the data to the ordinate intercept at $\Omega^0_B(x) = 0.43$. (b) The corresponding plot of $\Omega_A(x)$ defined by eq. (11a) versus $\bar{c}^*(x) = \bar{c}(x) - c_B(x)$. The extrapolated plot intercepts the ordinate axis at $\Omega^0_A(x) = 0.82$.

spanning the entire distribution in each case are reported. These results were analysed according to eqs. (9)–(11) to obtain the molar concentrations of unbound reactnats shown in the third and fourth columns of table 1. The extrapolation of $\Omega_B(x)$ to find $m_B(x_F)$ and of $\Omega_A(x)$ to find $m_A(x_F)$ are illustrated in fig. 1 which refers to the first experiment. For the second experiment, analogous extrapolations yielded $\Omega^0_B = 0.25$ and $\Omega^0_A = 0.40$ appropriate to the reference point $x_F = 6.9235$ cm, $\bar{c}(x_F) = 3.295$ g/liter. Columns five and six of table 1 report values of $P_A(x)$ and $P_B(x)$ calculated as described in the theoretical section for the cross-linking model with $f = 4$ and $k = 0.14 \times 10^4$ M^{-1}. Column 7 gives the corresponding calculated values of $\bar{c}(x)$, found with the use of eqs. (1), (2) and (12), which are compared with the experimental values by the deviations shown in column eight. It is seen from the reported standard deviations that these values of f and k for the cross-linking pattern suffice to describe both sets of sedimentation equilibrium results within available experimental precision. The larger deviation of ± 0.039 g/liter corresponds to a net fringe displacement of approximately 40 microns and is within the error generated in determining $\bar{c}(x)$ at the meniscus and in the extrapolation of $\Omega_B(x)$ and $\Omega_A(x)$. It could also be noted that the results of the first experiment were also fitted satisfactorily with standard deviations of less than ± 0.015 g/liter by the following sets of values of f and k, $(3, 0.19 \times 10^4$ M$^{-1})$, $(5, 0.11 \times 10^4$ M$^{-1})$ and $(6, 0.09 \times 10^4$ M$^{-1})$. However, these sets were inconsistent with the results of the second experiment, (where the extent of reaction indicated by values of P_A and P_B is greater), as indicated by standard deviations in excess of 0.36 g/liter. It is concluded on this basis that the value $f = 4$ for the cross-linking model is indicated by the sedimentation equilibrium results, a point which receives further support later in relation to binding results. In the latter connection, it could be noted immediately that values in excess of 2 were found for the binding function, which eliminated the necessity of considering the possibility that $f = 2$. The last two columns of table 1 summarize the analysis of the same values of $m_A(x)$ and $m_B(x)$, reported in columns three and four, in terms of the multiple binding model for which eqs. (12) and (13) are appropriate. Again acceptable standard deviations were found for both experiments, but in this case with $f = 4$, $k_A = 0.35 \times 10^4$ M^{-1}. It follows that analysis of sedimentation equilibrium results obtained previously [14] and in this work has not permitted characterization of the system in terms of a unique model, but it has for the first time provided descriptions of the results in relation to two possible models both now delineated by two parameters, (f, k) and (f, k_A).

Attention was now directed to binding results obtained by frontal gel chromatography. First, the values of $\bar{m}_A$ and m_B reported in table 1 of ref. [9] were used to calculate values of r from eq. (15) with $f = 4$ and $k = 0.14 \times 10^4$ M^{-1}, and from eq. (14) with $f = 4$ and $k_A = 0.35 \times 10^4$ M^{-1} (table 1). In the former case, referring to the cross-linking model, the standard

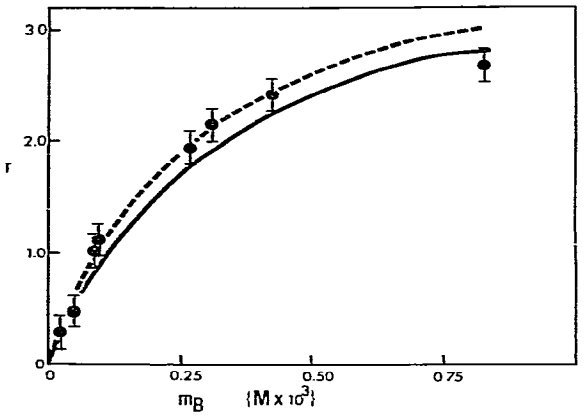

Fig. 2. Binding results obtained by frontal gel chromatography of mixtures of ovalbumin and lysozyme in pH 6.80 phosphate buffer at 25°C in which the ovalbumin concentration was held fixed at 0.22×10^{-4} M. The solid line was calculated for the cross-linking model using eqs. (15b) and (15c) with $f = 4$, $k = 0.14 \times 10^4$ M^{-1}. The broken curve was calculated for the multiple binding model using eq. (14) with $f = 4$, $k_A = 0.35 \times 10^4$ M^{-1}.

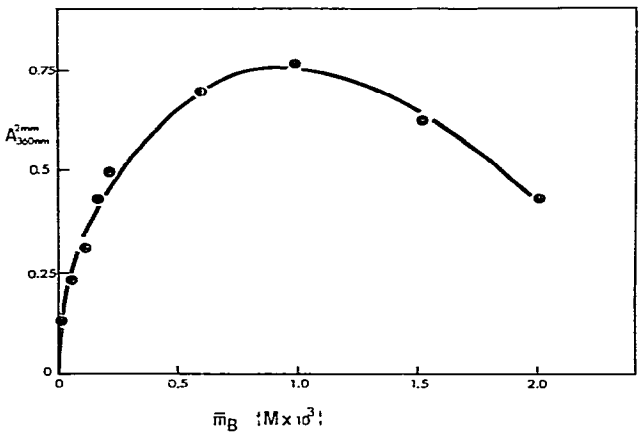

Fig. 3. A precipitin curve obtained on mixing pre-dialysed solutions of ovalbumin and lysozyme at pH 6.80 in 0.02 I phosphate buffer. The final concentration of ovalbumin in each mixed solution was 0.67×10^{-4} M ($\bar{m}_A$) and the abscissa shows the final concentration of lysozyme ($\bar{m}_B$) in the mixtures.

deviation (eleven comparisons) of experimentally determined and calculated r values was ± 0.09, while for the multiple binding model the value was ± 0.11. Both of these standard deviations are within the assessed error of ± 0.15 for r. In the present work, a series of similar experiments were conducted at a fixed value of $\bar{m}_A = 0.22 \times 10^{-4}$ M and with a range of values of $\bar{m}_B$ designed to encompass values of r larger than previously investigated. The results are summarized in fig. 2 together with theoretical curves calculated for the cross-linking and multiple binding models using eqs. (15) and (14), respectively, with the appropriate values of f and k (or k_A) reported in table 1. It is evident that either model provides a reasonable description of the experimental points when account is taken of the error bars, a finding which substantiates the analysis of sedimentation equilibrium results. Again, a value of $f = 4$ is indicated both for the multiple binding model by least squares fitting of the results to eq. (14) and for the cross-linking pattern for the following reason. It has been shown [7] that the limiting value of r as $m_B \to \infty$ is f and thus the fit of eq. (15) to the highest value of r observed provides the most sensitive guide to f. For $f = 4$. it is seen from fig. 2 that the deviation between experimental and calculated values is 0.12, while a similar comparison for $f = 3$, 5 and 6 yielded deviations of 0.4, 0.6 and 0.9, respectively.

The question now arises whether a choice may be made between the two models under discussion. Fig. 3 presents turbidimetric results obtained with a series of solutions in which the ovalbumin concentration, $\bar{m}_A$, was held fixed at a value of 0.67×10^{-4} M and the lysozyme concentration, $\bar{m}_B$, was varied from 1.39×10^{-5} M to 2.08×10^{-3} M. The striking feature of these results is the appearance of a maximum in the curve. Such an effect is possible in multiple binding, since it may be shown that $d\bar{m}_{AB_j}/d\bar{m}_B = 0$ when $m_B = j/k_A(f - j)$. This relation with $j = 1, 2, 3$ and $f = 4$, $k_A = 0.35 \times 10^4$ M^{-1} permits definition of the values of m_B at which the concentrations of the postulated complexes, AB, AB_2 and AB_3, respectively, attain maximal values (the fully saturated complex AB_4 exhibits no such maximum in concentration). In turn, eqs. (13a) and (13b) may be solved to yield the corresponding values of $\bar{m}_B$, 0.2×10^{-3} M, 0.4×10^{-3} M and 1.0×10^{-3} M, at the maxima. The latter value is seen to correspond quite well to the abscissa position of the maximum in fig. 3 and thus one explanation of the precipitin effect is that the

complex AB_3 is relatively insoluble. Admittedly, this is a somewhat hazardous postulate since this complex is neither large in size nor electrically neutral. If now the cross-linking model is considered in relation to fig. 3, we note that it has been shown [7] that those complexes characterized by $j/i = f/2$ attain their maximal concentrations at the same value of m_B ($= 1/2k$) as the maximum value of the extent of reaction. Such complexes in the ovalbumin–lysozyme system bear only a small net charge (approximately + 1) and are of considerable size at high values of i. It would not, therefore, be unreasonable to expect on this basis that the maximum in the precipitin curve would arise in the vicinity where $m_B = (1/0.28 \times 10^4)$ M which corresponds via eqs. (25a) and (25b) of ref. [7] to $\overline{m}_B = 0.5 \times 10^{-3}$ M, an abscissa position in fig. 3 in the region of the broad maximum. It now becomes evident that a measurement of the apparent weight-average molecular weight of the system near an initial composition corresponding to the predicted maximum for either model may provide a suitable means of distinguishing between them; for in this region the postulated systems differ significantly in the sizes of complexes predicted. Accordingly, an Archibald experiment [26,27] was performed with $\overline{m}_A = 0.67 \times 10^{-4}$ M (as in fig. 3) and $\overline{m}_B = 0.3 \times 10^{-3}$ M, selected as a compromise between the need to be near the maximum in fig. 3 but sufficiently distant from it to avoid a composition change inherent on maximum precipitation. The experimentally determined apparent weight-average molecular weight was 65 000 ± 6 000 which compares favorably with that of 61 000 calculated for the cross-linking model, but unfavorably with that of 50 000 estimated for the multiple binding model. These calculated values were obtained by computing m_A and m_B from the selected $\overline{m}_A$ and $\overline{m}_B$ values using eq. (25a) and (25b) of ref. [7] and eqs. (13a) and (13b) of this work. Expressions are then available [7,15] for the calculation of m_{AB_j} and $m_{A_iB_j}$ which may be utilized directly in the standard way to obtain the weight-average molecular weights, account being taken of the contributions of unbound A and B. On this basis it would appear that the cross-linking interaction pattern with $f = 4$ and $k = 0.14 \times 10^4$ M^{-1} better describes the equilibria operating in mixed solutions of ovalbumin and lysozyme, an interpretation consistent with sedimentation equilibrium results (table 1), binding results (fig. 2) and reasonably in accord with the precipitin effect (fig. 3).

5. General remarks

The development of the interpretation of results obtained with the ovalbumin–lysozyme system in terms of both multiple binding and cross-linking interaction patterns has highlighted the fact that certain sets of experimental findings may well find explanation on the basis of either scheme. The danger, therefore, exists that the familiar theory of multiple binding [15,16,25,28] involving only complexes of the type AB_j may be utilized solely in the interpretation of interactions between dissimilar molecules. No doubt this is a reasonable approach when specific interactions occur, for example, between an enzyme and its competitive inhibitors or effectors. However, it is inappropriate to investigations involving bivalent ligands [1–8,29] and may be seriously questioned in cases where electrostatic attractive forces contribute significantly to the total free energy of the system: this is particularly relevant in situations where by reason of stoichiometry no electrically neutral complex can be formed. In the latter case, of which the ovalbumin–lysozyme system is but one example, it may indeed be naive to view solution composition solely in terms of a limited number of complexes comprised of a single acceptor molecule with varying numbers of ligand molecules attached to it. A more realistic view seems to be that a spectrum of forces are operative, a situation which has been formalized in thermodynamic terms in this work by invoking a large number of reversible reactions involving the formation of the extensive array of complexes A_iB_j ($i = 1, 2, \ldots, \infty$; $j = (i-1), i, \ldots, i(f-1)+1$).

At first sight, the problem of formulating experimentally useful relations based on this model may seem formidable. However, it has now been demonstrated with the use of reacted site probability functions that a variety of such expressions are available and useful when information is available on $m_A(x)$ and $m_B(x)$ as in sedimentation equilibrium analysis (fig. 1 and table 1) or on r and m_B as in binding studies (fig. 2). Moreover, relations exist which permit the calculation (for known values of $\overline{m}_A$, $\overline{m}_B$, f and k) of the concentrations of the complexes A_iB_j

and hence comment is possible on weight-average quantities and on precipitin effects. Certainly, the present treatment of the problem has been based on certain simplifying assumptions, notably that the ligand may be viewed as bivalent, that a single site binding constant suffices to describe all equilibria and that non-ideality effects are negligible. It is of some reassurance that results on the ovalbumin–lysozyme system may be satisfactorily formalized on this basis in terms of two parameters, f and k, and it seems entirely possible that the basic theory of cross-linking interactions may be extended when the need arises. For the present, it is hoped that the theory developed herein and the illustration of its application will be useful in examining, by a more diverse range of techniques than hitherto utilized, other systems of the cross-linking type.

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